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Pertanika is an international peer-reviewed journal devoted to the publication of original papers, and it serves as a forum for practical approaches to improving quality in issues pertaining to tropical agriculture and its related fields. *Pertanika* Journal of Tropical Agricultural Science which began publication in 1978 is a leading agricultural journal in Malaysia. After 29 years as a multidisciplinary journal, the revamped *Pertanika* Journal of Tropical Agricultural Science (JTAS) is now focusing on tropical agricultural research. Other *Pertanika* series include *Pertanika* Journal of Science and Technology (JST) and *Pertanika* Journal of Social Sciences and Humanities (JSSH).

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Review Article

Streptococcosis in Tilapia (*Oreochromis niloticus*): A Review

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ABSTRACT

Tilapia (*Oreochromis niloticus*) is a hardy, most cultured freshwater fish in the world. It has been contributing to the world aquaculture since the ancient Egyptian days and remains a major freshwater fish species to be cultured. Although tilapias are more resistant to unfavourable water quality than other freshwater fish, tilapias have been reported to succumb to infection by *Streptococcus*, which was first observed among the populations of rainbow trout (*Oncorhynchus mykiss*) farmed in the Shizouka Prefecture in Japan in April 1957. Since then, the disease that is also known as 'pop eye' has been reported in many other fish species throughout the world, contributing to an annual loss of approximately USD 150 million. Affected tilapia shows loss of appetite, spine displacement, haemorrhages in the eye, corneal opacity, haemorrhages at the base of the fins and in the opercula. The most prominent signs are uni- or bi-lateral exophthalmia (also known as "pop-eye"), distended abdomen and erratic swimming. Control is mainly through implementing some preventive measure and antibiotic therapy, while vaccination is generally not effective in preventing *Streptococcus* outbreaks in tilapias.

Keywords: *Streptococcus*, tilapia, infection

INTRODUCTION

The tilapias are freshwater fish that belong to the family Cichlidae, and they are exclusively associated with Africa and Middle East (Trewaves, 1983). The Nile tilapia (*Oreochromis niloticus*) is one of the first fish species to be cultured in the world. Illustrations from Egyptian tombs suggested that the Nile tilapias had been cultured more than 4,000 years ago, i.e. about 1000 years before carp was introduced into China (Balarin & Hatton, 1979). Tilapias have been called the "Saint Peter's fish" in reference to biblical passages about the fish being fed to the multitudes (Popma & Masser, 1999). Pillay (1990) reported that tilapias were introduced into many tropical, sub-tropical and temperate regions of the world during the second half of the 20th century.

In the world where captured wild fisheries are becoming increasingly depleted, tilapias offer a possibility of commercialization because of their superior culture adaptability. According to FAO (2004), tilapias (*Oreochromis* sp.) are among the most cultured fish worldwide. In fact, the production of tilapias made the fish one of the most important species for the 21st century aquaculture (Fitzsimmons, 2000) which also rose commercially in more than 100 countries (Shelton & Popma, 2006).

The estimated global tilapia production for the year 2000 was 1.5 million metric tonnes

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(MT) compared to merely 28,260 metric tonnes in 1970. Similarly, the value of the farmed tilapias also increased from about USD 154 million in 1984 to USD 1,800.7 million in 2002 (FAO, 2004). China alone produced 706,585 MT of farmed tilapia (representing 50% of the total world production), followed by Egypt (167.7 MT), the Philippines (122.4 MT), Indonesia (109.8 MT), Thailand (100.6 MT), Taiwan (85.1 MT), Brazil (42 MT), Colombia (24 MT), Malaysia (20.8 MT), and Laos (20.8 MT). The farmed tilapias had exceeded 2 MMT in 2004 worldwide (FAO, 2004; El-Sayed, 2006).

Tilapias have good characteristics for farming and are now so domesticated that this fish species has earned the title "aquatic chicken". Moreover, tilapias are fast-growing with firm, white flesh, and able to survive in poor water conditions, eat a wide range of food types, breed easily with no need for special hatchery technology (Nandlal & Pickering, 2004), as well as feed at the base of the aquatic food web (Beveridge & Baird, 1998). Tilapias are tough and can tolerate a wide range of environmental conditions; therefore, little environmental modification with low technology system is needed for culturing tilapias (Pullin & Lowe-McConnell, 1982; Welcomme, 1988; Beveridge & McAndrew, 1998; Nandlal & Pickering, 2004).

Initially, tilapias were considered to be more resistant to bacterial, parasitic, fungal, and viral diseases compared to other species of cultured fish. In more recent times, however, tilapias have been found to be susceptible to both bacterial and parasitic diseases. Common tilapia pathogens include *Streptococcus* sp*., Flavobacterium columnare, Aeromonas hydrophila, Edwarsiella tarda, Ichthyophitirius multifillis, Tricodhina* sp., and *Gyrodactylus niloticus* (Klesius *et al*., 2008). It is important to note that streptococcal infections have become a major problem in tilapias farming and contributed to severe economic losses (Shoemaker & Klesius, 1997). *Streptococcus iniae* and *Streptococcus agalactiae* are the major bacterial species that affect the production of tilapias in the world (Evan *et al*., 2006a).

The aims of this paper are to review the current information on streptococcosis, including epidemiology, main water quality that contributes to the disease, mode of transmission, pathogenesis, as well as disease diagnosis, and control measures in farmed tilapias.

STREPTOCOCCUS AND STREPTOCOCCOSIS

Streptococcosis is a disease that develops following the infection by *Streptococcus* sp. They are spherical or ovoid in shape and 0.5-2.0 µm in diameter. They occur in pairs or chains when grown in liquid media, are non-motile, non spore-forming and Gram-positive. A major identification feature of *Streptococcus* is that it is Gram-positive that appears purple/blue when stained using a procedure called a Gram stain. On the contrary, most of the common disease-causing bacteria of fish are Gram-negative and appear pink with Gram stain (Yanong & Floyd, 2002). It is facultatively anaerobic, requiring nutritionally rich media for growth and commonly attacks red blood cell to produce greenish discolouration (α-hemolysis) or complete clearing (β-hemolysis) on blood agar. In addition, it is also a type of bacteria that are fermentative in metabolism, producing mainly lactic acid, but without gas and catalase negative (Holt *et al*., 1994).

In fish, it was initially described among the populations of rainbow trout (*Oncorhynchus mykiss*) farmed in the Shizouka Prefecture in Japan in April 1957 (Hoshina *et al*., 1958). After that, Robinson & Meyer (1966) reported two epizootics, both involving infections of golden shiner (*Notemigonus crysoleucas)* with *Streptococcus*. Meanwhile, Plumb *et al*. (1974) isolated *Streptococcus* sp. from over 50% of the diseased fish during an epizootic in the estuarine bays along the Florida, Alabama, and the Gulf Coast of Mexico in the United States in 1972. In fish, *Streptococcus* spp. has been reported to cause considerable morbidity and mortality worldwide. Estimated losses were around USD 150 million annually in 2000 and these further increased to USD 250 million annually in 2008 (Klesius *et al*., 2000; Klesius *et al*., 2008).

Streptococcus iniae was first isolated from a skin lesion of a captive Amazon River fish, *Inia geoffrensis* (Pier & Madin, 1976). Since then, the bacterium has been reported in many species of fresh, estuarine and marine fish species from 15 countries in 6 continents, including Africa, Asia, Australia, Europe, as well as North and South Africa. The susceptible fish include ayu (Kitao *et al*., 1981), barramundi (Bromage *et al.*, 1999), coho salmon (Eldar *et al*., 1995a), European seabass (Zlotkin *et al*., 1998), grey mullet (Eldar *et al*., 1995a), grouper (Kvitt & Colorni, 2004), rainbow trout (Eldar *et al*., 1994), red drum (Shen *et al*., 2005), snapper (Ferguson *et al.*, 2000), silver bream (Bromage & Owen, 2002), tilapia (Klesius *et al.*, 2006a), and yellowtail (Kaige *et al*., 1984).

Group B *Streptococcus agalactiae*, another emerging fish pathogen, has been shown to cause significant morbidity and mortality among a variety of freshwater and saltwater fish species throughout the world (Robinson & Meyer, 1966; Plumb *et al*., 1974; Evans *et al.,* 2002). *Streptococcus agalactiae* was first reported in the captive freshwater shiners in 1966 (Robinson & Meyer, 1966). Recently, this particular bacterium has been reported in fish from 7 countries in 3 continents, namely the United States (North America), Israel, Japan, Kuwait and Thailand (Asia), Honduras (Central America), and Brazil (South America). This pathogen has also been isolated from 17 fish species including rainbow trout, seabream, tilapia, yellowtail, *catfish* sp., croaker, killfish, *menhaden* spp., *mullet* spp. and silver pomfret (Wilkinson *et al*., 1973; Plumb *et al*., 1974; Rasheed & Plumb, 1984; Elliot *et al.*, 1990; Baya *et al*., 1990; Eldar *et al*., 1995a; Vandamme *et al*., 1997; Evans *et al.*, 2002; Duremdez *et al*., 2004; Suanyuk *et al*., 2005; Salvador *et al*., 2005; Evans *et al.*, 2006a; Kim *et al*., 2007; Garcia *et al.*, 2008).

Streptococcus spp. is considered a diverse group of bacteria that possess the capacity to infect a wide range of hosts. Among other, *S. iniae* has been isolated from humans with bacteraemia, cellulitis, meningitis, and osteomyelitis (Facklam *et al*., 2005). The source of human infections has been associated with the preparation of *S. iniae* infected tilapias for cooking (Lehane & Rawlin, 2000). *S. agalactiae* is the causative agent of neonatal meningitis, sepsis and pneumonia in human (Baker, 1980). It has been isolated from chickens, cattle, camels, dogs, bottlenose dolphins, horses, emerald monitors, cats, fish, frogs, hamsters, humans, mice, monkeys, and nutria (Wilkinson *et al.*, 1973; Elliott *et al*., 1990; Evans *et al.*, 2002; Zappulli *et al.*, 2005).

Transmission

Many studies have been carried out to reveal the transmissions of *Streptococcus* sp. According to Nguyen *et al*. (2002), the newly introduced fish is the most important factor that introduced *S. iniae* and *S. agalactiae* into the farm. The bacteria are excreted in the faeces of infected fishes, survive in the water and be infectious to other healthy fish (Nguyen *et al.*, 2002). Besides, using the infected thrashed fish as feed is believed to be responsible for the outbreaks of streptococcosis among flounder in Korea (Kim *et al.*, 2007). Similarly, an experimental study revealed that cohabitation of dead or infected fish with healthy fish resulted in the infection of the healthy fish. Meanwhile, the horizontal transmission of the pathogens between fish is believed to be the most common mechanism of dissemination. A study by Xu *et al.* (2007) showed that the infection by this particular pathogen could occur through wounds and abrasions of the skin. This mechanism usually involved in fish that were cultured in high densities. Furthermore, the transmission of *Streptococcus* between different species of wild and cultured fish, within the same aquatic environment, is likely to occur (Evans *et al.*, 2002). This is because wild fish and fish cultured nearby have been found to be infected with the same *S. iniae* strains in Israel (Colorni *et al.*, 2002). Similarly, Bromage & Owen (2002) reported that the fish cohabiting barramundi pens had the same *S. iniae* strains as the barramundi. In addition, the transmission among the species of reef fish has also been reported in the Caribbean (Ferguson *et al*., 2000).

Pathogenesis

Infection by *Streptococcus* leads to various clinical signs, which include haemorrhages at the gill plate, loss of appetite, spine displacement, haemorrhages in the eye, corneal opacity, and haemorrhages at the base of the fins and in the opercula. The most prominent signs are uni- or bi-lateral exophthalmia, also known as "pop-eye", and distended abdomen. The post-mortem examinations of the affected fish revealed the presence of blood-tinged fluid in the body cavity, enlarged and reddened spleen, pale but enlarged liver, as well as inflammations around the heart and kidney. Meanwhile, hemorrhagic lesions were observed on the skin (Bullock, 1981; Yanong & Floyd, 2002; Salvador *et al*., 2005). Other clinical signs include darkening of the skin and erratic swimming, which is either spiralling or spinning just below the surface of water. In some cases, however, the affected fish showed no obvious clinical signs before death and the mortality is mainly due to septicaemia and infection of the brain and nervous system (Barham *et al.*, 1979; Yanong & Floyd, 2002).

Buchanan *et al.* (2005) identified enzyme phosphoglucomutase as the virulence factor for *S. iniae*. This enzyme inter-converts glucose-6-phosphate and glucose-1-phosphate which play important role in the production of *S. iniae* polysaccharide capsules. Unlike *S. iniae*, the regulatory proteins and enzymes associated with cell surface metabolism have been revealed as the virulence factors for *S. agalactiae.* Therefore, the removal of the genes that are involved in these functions can reduce the virulence. Fuller *et al*. (2002) found that the virulence factor could also be caused by the gene that is associated with β-hemolysis. However, additional research should be carried out to identify and characterize the genes and the virulence factors that regulate their expression.

FACTORS CONTRIBUTING TO THE DEVELOPMENT OF STREPTOCOCCOSIS

The presence of the pathogen in the environment of the fish is inadequate to cause a disease outbreak. Other factors usually come into play, such that either the pathogen has an advantage over the host or the immune system of the host is compromised in some ways, increasing its susceptibility to the pathogen. This phenomenon is often precipitated by "stress" (Yanong & Floyd, 2002). Therefore, stress often plays a significant role in the outbreaks of infectious disease in fish populations. Some stressors that have been associated with the Streptococcal outbreaks include high and low water temperatures, high salinity and alkalinity (pH>8), low dissolved oxygen concentration, poor water quality (such as high ammonia or nitrite concentrations), high stocking densities, as well as harvesting and handling effects (Chang & Plumb, 1996; Bunch & Bejereno, 1997; Bowser *et al*., 1998; Yanong & Floyd, 2002).

Meanwhile, water quality parameters can contribute to the development of disease. It is a well-known fact about the intolerance of tilapias to low temperatures, which is a serious constraint for commercial culture in temperate regions (Chervinski, 1982; Cnaani *et al.*, 2000). Reproduction of tilapia is best in water temperatures above 27°C, but it does not occur when water temperature is below 20°C. In sub-tropical regions with a cool season, the numbers of fry produced are decreased when daily water temperature averages less than 24°C. It was concluded that the optimal water temperature for the growth of tilapias is between 29°C and 31°C (Popma & Masser, 1999), but a water temperature of ≥31°C predisposes tilapias to the outbreaks of *Streptococcus agalactiae* infection (Evans *et al.*, 2006a; Amal *et al.*, 2008).

Oxygen is the first limiting factor for growth and well-being of fish. Fish require oxygen for respiration, which physiologists express as the mg of oxygen consumed per kilogram of fish per hour (mgO2/kg/h). Although tilapia can survive acute low DO concentrations of less than 0.3 mg/L for several hours, tilapia ponds should be managed to maintain the DO concentrations above 1 mg/L. Metabolism, growth, and disease resistance are depressed when DO falls below this level for a prolonged period (Popma & Masser, 1999), predisposing tilapias to streptococcosis.

Moreover, it is a well-known fact that increasing water temperature will reduce the rate of DO in the water. The high water temperature also leads to increased respiration rate and oxygen consumption by tilapias because of the high metabolism rate. This further increases the demand for oxygen by tissues. Therefore, dissolved oxygen concentration greater than 5 ppm is required for a good growth of tilapias (Swann, 1992; El-Sayed, 2006).

Other than water temperature and dissolved oxygen, massive mortality of tilapia occurs within a few days when fish are suddenly transferred into water without ionized ammonia concentration greater than 2 mg/L. Meanwhile, a prolonged exposure for several weeks to un-ionized ammonia concentration greater than 1 mg/L in water with low dissolved oxygen predisposes tilapias to diseases including streptococcosis. In fact, the prolonged exposures to 0.2 mg/L of un-ionized ammonia concentration are found to be detrimental to fish (Popma & Masser, 1999). Ahmed *et al.* (1992) have found that Nile tilapias exposed to ammonia had lower number of red blood cells leading to haemolytic anaemia and significant reduction in blood oxygen content, which enhances ammonia toxicity.

Nitrate is relatively non-toxic to tilapias. However, a prolonged exposure to elevated levels of nitrate may decrease the immune response and induce mortality (Plumb, 1997). Inversely, nitrite is highly toxic to tilapias because it disturbs the physiological function of the fish and leads to growth retardation (El-Sayed, 2006). Nitrite may enter the bloodstream passively as nitrous acid and freely diffuses across the gill membranes of the fish. After entering the bloodstream, nitrite oxidizes the iron in the haemoglobin molecule from ferrous state (Fe^{2+}) to ferric state (Fe^{3+}) and the resulting product is called methemoglobin. Since methemoglobin is incapable of reversibly binding with oxygen, exposures to nitrite can cause considerable respiratory distress because of the loss in blood oxygen-carrying capacity (Boyd & Tucker, 1998).

In general, tilapias can survive in pH ranging from 5 to 10, but they do best in a pH range of 6 to 9 (Popma & Masser, 1999). On the contrary, low water pH leads to behavioural changes, damage of the gill epithelial cells, reduction in the efficiency of the nitrogenous excretion and increased mortality. Wangead *et al*. (1998) reported that fingerlings and adult tilapias exposed to pH 2-3 showed rapid swimming and opercula movement, surfacing and gulping of air, as well as lack of body position and mass mortality within 1-3 days. A study by Chen *et al*. (2001), on the other hand, showed that tilapias exposed to high water pH for 7 days decreased ammonia excretion, but increased urea nitrogen excretion. Bonga *et al*. (1987) revealed that slow acclimatization of tilapias to low or high pH levels might enable the fish to withstand long-term exposures to the acidic or alkaline water. Thus, farmers should be aware of the sudden change in water pH to prevent stress on their cultured tilapias that may lead to disease outbreaks (Bonga *et al.*, 1987).

Diagnosis

The presence of typical clinical signs and demonstration of Gram-positive cocci from the brains, kidneys, eye or other internal organs constitutes a presumptive diagnosis of streptococcosis. The causative bacteria are best detected in the brains of diseased fish (Sugiyama & Kusuda, 1981). Streptococcal infection should be highly suspected if the affected fish exhibit abnormal swimming behaviour, pop-eye, haemorrhages, and rapid severe mortalities, while Gram-positive cocci are found in brain, kidney, and/or other organs. A confirmed diagnosis requires culture of internal organs, specifically the brain and kidney, followed by identification of the bacterium (Yanong & Floyd, 2002).

To recover the streptococci is apparently straightforward; bovine blood tryptose agar (Naude, 1975; Roode, 1977; Boomker *et al.*, 1979), brain heart infusion agar (BHIA) (Minami *et al.*, 1979, Ugajin, 1981), Todd-Hewitt broth,

nutrient agar supplemented with rabbit blood (Kitao *et al*., 1981) are suitable media for culture. Inoculated media should be incubated at 22-37°C for up to 48 hours before the "dull grey" colonies of approximately 1-2 mm in diameter develop. This pathogen is easily grown on BHIA (Plumb *et al*., 1974). Beside that, it also can grow on tryticase soy agar supplemented with 0.5% glucose, Todd-Hewitt broth agar (THBA), and horse blood agar (Kitao, 1982).

A single colony from pure culture should be Gram-positive cocci, oxidase, and catalase negative and either non-hemolytic or β-hemolytic on agar plate. The carbohydrate group antigen test should be also among the first presumptive test performed. The only group B streptococcal species is *S. agalactiae*. In contrast, *S. iniae* does not have a carbohydrate group antigen. If the streptococci hydrolyze starch, it is also presumptive test for *S. iniae* (Evans *et al*., 2004). Meanwhile, the biochemical and other identification tests have been fully described elsewhere (Shoemaker *et al*., 2001; Evans *et al*., 2002).

Rapid kits such as API 20E, API Rapid Strep 32, and API CH50 could not be used to identify *S. iniae* because this particular bacterium is not included in the database system. However, these rapid kits can be used for the identification of *S. agalactiae* and other *Streptococcus* spp. (Evans *et al*., 2006a). Jayarao *et al*. (1991) compared the identification systems between Vitek-Gram positive and API Rapid Strep 32 system and found that 93% of *S. agalactiae* isolates could be identified using both the kit systems. A comparison between API Rapid Strep 32 System and Biology system using Gram-positive plates revealed that both the systems produced 100% identification of the *S. agalactiae* isolates (Evans *et al*., 2006b). However, the Biology system using Gram-positive plates was able to correctly identify approximately 70% of *S. iniae* (Roach *et al*., 2006).

Molecular diagnosis using the PCR technique is useful to identify streptococcus. Many of the PCR techniques make use of the 16S rRNA gene as the molecular marker for the identification of *S. iniae* (Zlotkin *et al*., 1998). Besides, a PCR technique using 16S-23S ribosomal DNA intergenic spacers was found to be useful for the identification of *S. agalactiae* from fish (Berridge *et al*., 2001). However, the results of the PCR assay should be supported by presumptive techniques to ensure the accuracy of the detection.

Klesius *et al.* (2006a) developed an indirect fluorescent antibody technique (IFAT) based on a highly specific monoclonal antibody for a rapid detection of *S. iniae*. The olfactory epithelium of naturally infected tilapias was demonstrated to be a reliable, sensitive and non-lethal sample site for the detection and identification of *S. iniae*.

Controls

Chemotherapy

Several drugs have been tested for the treatment of streptococcosis. Among other, Darwish & Griffin (2002) found that oxytetracycline was effective in controlling *S. iniae* in blue tilapias (*O. aureus*). Oxytetracycline was incorporated into the feed at 0, 25, 50, 75, and 100 mg/kg body weight. The 75 and 100 mg doses significantly increased the survival of the infected fish from 7% to 85 and 98%, respectively.

Some reports concluded that erythromycin is effective against streptococcal infections in cultured yellowtails (Shiomitsu *et al.*, 1980) and rainbow trout (Kitao *et al*., 1979) at doses of 25-50 mg/kg/day for 4 to 7 days. Doxycycline, oxytetracycline, kitasamycin, oleandomycin, josamycin, and lincomycin have also been used to control streptococcosis in the cultured yellowtail in Japan (Kitao *et al.*, 1979). Doxycycline, at 20 mg/kg/day for an undetermined duration, has also been advocated (Nakamura, 1982). Similarly, a novel fisheries therapeutant, i.e. sodium nifurstyrenate, dosed at 50 mg/kg body weight of fish/day for three to five days has been proven to be successful in treating streptococcosis when incorporated with feed (Kashiwagi *et al.*, 1977).

Meanwhile, streptococcal infections respond to antibiotic therapy, but the disease cannot be legally controlled with antibiotics all the way to the market because the withdrawal period for all

effective antibiotics is longer than it takes for the streptococcal infection to return. Furthermore, it is only a matter of time before *Streptococcus* develops resistance to the antibiotics. In fact, streptococcal strains at several facilities have already developed resistance to some antibiotics (Darwish & Hobbs, 2005). Therefore, antibiotic treatment is generally ineffective and the need of proper vaccine has become a must (Klesius *et al*., 2000).

Preventive measures

If causative streptococci are present in the mud and water throughout the aquatic environment, avoidance is not an easy or practical means of disease prevention. However, purchasing specific pathogen-free stock, quarantining new arrival fish stock, reducing overcrowding, avoiding overfeeding, keeping separate water supplies for culture systems, minimizing unnecessary handling or transportation, removing dying and dead fish frequently, feeding pathogen free ration, and keeping excellent sanitary conditions will reduce the risks of disease outbreak (Inglis *et al*., 1993; Klesius *et al*., 2008). Furthermore, periodic cleaning and disinfections of all the production units and equipment should be done to decrease the transmission of pathogens. Maintaining good water quality in the systems is also necessary (Klesius *et al.*, 2008).

Management control by 'break-cycle' has been suggested (Amal *et al.*, 2008). April-June is a critical period in tilapia culture because of the high water temperature, while fish that weigh 150-300 gram are in critical condition. Huge and slow flow water bodies are critical situations for development streptococcosis in Malaysia. Farmers are advised to manage the cultured fish so that harvesting of adult fish of more than 200 gram can be done before the coming critical period of April-June and to ensure that only fish of less than 100 gram are available in the cage at the critical months of April-June. Nevertheless, farmers who still keep fish of 150-300 gram during the critical period of April-June are advised to reduce overcrowding by re-distributing the fish in cages.

Vaccines

A vaccine is a preventive tool used in a heath management strategy for controlling infectious disease (Klesius *et al.*, 2006a). In aquaculture, the development and use of vaccines are now making rapid progress to achieve their full potential as effective disease prevention tools. The objective of vaccination is to provide a strong immune response to an administered antigen that is able to produce acquired long-term protection against a pathogen. Killed and modified live vaccines have been developed for use in aquaculture. The type of immunity needed, antibody or cell mediated, against a particular pathogen are among the deciding factors in the development of a vaccine. Killed vaccines are usually administered by intraperitoneal (IP) or intramuscular injection (IM) of individual fish. Injection is the least cost effective in terms of labour and time. Meanwhile, killed vaccines are considered safer than the modified live vaccines, which may revert to virulence. Consequently, future trends may include oral delivery of vaccine, immersion delivery of killed vaccine, development of additional modified live vaccines and multivalent vaccines and improved vaccine adjuvants and immunostimulant. Vaccines prevent disease and mortality, but they may not completely eliminate streptococci in surviving fish (Klesius *et al*., 2008).

The first killed vaccine was developed to prevent losses in trout due to *S. iniae* infection in Israel (Eldar *et al*., 1997). The mortality of rainbow trout intraperitoneally (IP) immunized with formalin killed *S. iniae* vaccine was 5%, whereas in non-immunized rainbow trout, the mortality exceeded 50% in the field trial. Whole cell and bacterial protein vaccines were produced against *S. agalactiae* (Eldar *et al*., 1995b). A non-autogenous killed *S. iniae* vaccine supplemented with its extracellular products (ECP) was found to be effective in tilapia (Klesius *et al*., 1999). The mortality was reduced by 91.3% in tilapia immunized IP with this vaccine at 30 days post-experimental challenge with *S. iniae*. The molecular weight of the extracellular product was greater than 2kD. The relative percent survival was 95%

in 25 gram tilapia and 84.2% to 94.7% in 100 gram tilapia. Besides that, western blot analysis revealed predominant 54 and 55 kDa antigens in the extracellular products (ECP) of *S. agalactiae* (Pasnik *et al*., 2005). The results of the study provided a correlation between protection and antibody production against ECP and for the importance of the 55 kDa antigen for vaccine efficacy against *S. agalactiae*.

CONCLUSION

In conclusion, *Streptococcus* spp. (specifically *S. agalactiae* and *S. iniae*) are very pathogenic as they can affect many fish species in the world. In particular, Streptococcosis has been reported to occur in fresh, marine and brackish water fish; thus, it has caused millions economic losses of aquaculture in the world. Tilapias have become a perfect host for *Streptococcus* infection. Tilapia farmers should be advised and educated on a proper management of tilapias so as to prevent the outbreak and spread of the disease. In addition, water quality parameter plays an important role in tilapias farming. In more specific, an optimum water quality parameter should be maintained to prevent "stress" in fish which can lead to outbreaks of disease. The diagnosis of diseased and carrier fish can be made by using rapid and accurate immunological and molecular techniques. Although chemotherapy was not really suggested, good management practice and vaccination could be parts of the plan to prevent and control streptococcosis.

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Effects of Deficit Irrigation on Water Productivity and Maize Yields in Arid Regions of Iran

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ABSTRACT

Deficit irrigation in the Gavkhuni River Basin (GRB), Iran, is an effective method for alleviation of drought impacts on crop yields. Whilst it saves considerable amounts of water, it has little effect on crop yields. The effects of deficit irrigation on grain yield and yield components of maize were studied using two factors [namely, the variety at two levels (704 maize variety with 9354 kg ha^{-1} yield, and 647 maize variety with 8822 kg ha^{-1} yield) and irrigation at four levels (control, 100, 80, and 60% of water level use)] for three consecutive years. Significant differences ($P \leq 0.05$) were noticeable in grain yield, as well as depth and column of kernel among the irrigation treatments. In addition, the effects of cultivars on grain yield, 1000 kernel weight, number of kernel per ear row, number of kernel per column, and depth of kernels were insignificant. Nevertheless, the effects of irrigation treatments on 1000 kernel weight and number of kernel per ear row were not significant. Based on the results and considering the quantitative characteristics of the crop, it was established that for the deficit irrigating of maize, the 80% irrigation level (i.e. 80% of crop evapotranspiration) is the most advantageous treatment when water is not limited. However, when higher water productivity and the possibility of using the water saved are taken into consideration during severe drought conditions, 60% irrigation level treatment is recommended.

Keywords: Deficit irrigation, maize, yield components, water productivity

INTRODUCTION

The limited and/or expensive available water supply makes it impractical to irrigate the entire irrigable land area. Therefore, irrigators must decide between fully irrigating a small area for maximum production and reducing the depth of water applied per unit area in order to increase the area put under irrigation. The latter strategy is called deficit irrigation (DI), which will reduce reasonable crop yield per unit of land but increases the net return for the water applied. DI maximizes water productivity (WP), which is the main limiting factor (English, 1990). The determination and analysis of the agricultural WP index in Iran are essential to find suitable methods for better and economical use of water for agriculture. Thus, field data such as crop yield, different levels of water use, and irrigation

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management practices are necessary and pertinent to the formulating of water resources policies for optimal agricultural production and advancement in Iran.

It is important to note that maize is one of the important and strategic crops in Iran and its average yield in the country is 8.57 ton ha^{-1} while this is about 8 ton ha^{-1} across the study area (Anonymous, 2007). Tavakoli (1999) has shown that the yield reduction in Iran is much lower than the water reduction under the DI. Thus, the selection of a method for DI by farmers is important. Aghdaii & Sattar (2000) revealed a significant (direct) effect of the levels of irrigation water on maize yield ($P \leq 0.05$) and 100% irrigation level gave the maximum yield. Meanwhile, Emam & Ranjbar (2000) studied the effects of plant density and water stress on grain yield (GY) and water use efficiency of the maize hybrid, SC704. The results of their study showed that water use efficiency had increased in water stress and high crop density treatments. Oktem (2008) demonstrated that the relationships between fresh ear yield and irrigation level treatments were statistically significant ($P \leq 0.05$), and the yield decreased with increasing DI. However, the study showed that the number of marketable ears at 10% water deficiency was still high and acceptable for sweet corn in south-eastern Turkey. Chen *et al*. (2009) revealed that increase of irrigation amount resulted in more crop yields, but the water amount required to gain maximum WP was much less than that required for obtaining the maximum crop yield. Payero *et al*. (2008) showed that the differences in seasonal water requirements among irrigation depth treatments significantly ($P \leq 0.05$) affected dry matter production and yield components of maize. Moreover, water use efficiency was more sensitive to irrigation water and decreased explicitly with irrigation.

Zwart & Bastiaanssen (2004) reported that the range of crop WP of maize, based on a review of 84 literature sources, is very large (1.1–2.7 kg m^{-3}) and it thus offers new water management practices for increasing crop production with 20–40% less water resources. They concluded that in order to achieve optimum crop WP in water short regions, it would be wise to irrigate maize and wheat with less water. Geerts & Raes (2009), who had reviewed many research from around the world, confirmed that DI is successful in increasing WP for various crops without causing severe yield reductions. They further suggested that in regions where the available water supply limits agricultural production, farmers must select crops and irrigation strategies to maximize their crop yields and livestock production activities. Although a perception of WP is required to develop improved water management strategies, little is known about its application at the irrigation field level at the GRB. The vulnerability amongst farmers, at the tail-end of the GRB (Rudasht East and West networks) [*Fig. 1*] in spite of their larger farm possession, demands that an improved water management program is necessary at the head of irrigation networks (of the study area), in order to increase water equity. Furthermore, with an effective DI management in the study area, it is believed that it can contribute to improve the livelihood of the farmers.

The objectives of this study were:

- To determine the effect of water deficit (as quantified by different irrigation levels) on maize yields and yield components.
- To establish optimal water management strategies for maize in the GRB for the purpose of achieving more WP in limited water or water stressed environments.

MATERIALS AND METHODS

Research Location and Condition

The GRB (41,500 km^2) is a closed basin with no outlet to the sea (see *Fig. 1*). The research was conducted in the Nekuabad district of Isfahan state, which is located in the central part of the GRB. The Agricultural Research Centre (32°, 38' N, 51°, 22' E) is located at the altitude of 1649 m above the sea level. A large part of the

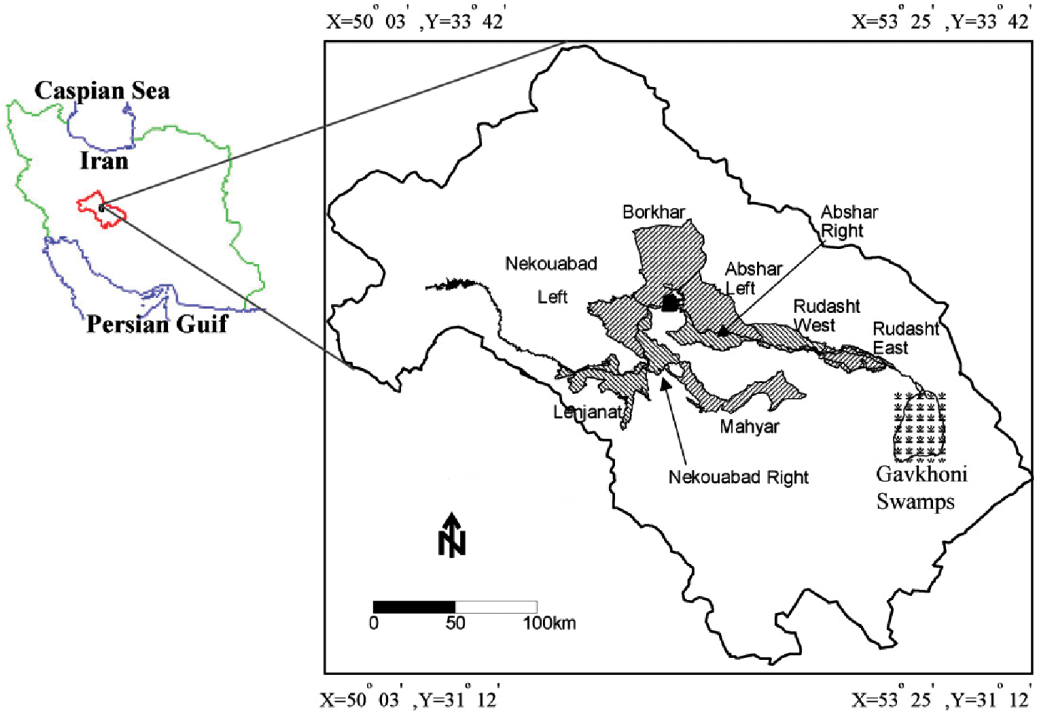

Fig. 1: Location of the study area and major irrigation networks in the GRB, Isfahan, Iran

basin has typical arid and semiarid climate with an average rainfall of 165 mm concentrated over the months of December to May, and whence it is almost impossible to have any economic form of agriculture without reliable irrigation. During the experimentation, there was a severe drought in the region and as well as throughout the whole country, i.e. when average rainfall declined to 48 and 70 mm in the years 2000-2001 and 2002-2003, respectively. The soil of the experimental area, according to USDA Soil Taxonomy 1994 (Anonymous, 1998), is of fine loamy, mixed, thermic, typiccalcigypside. At the soil depth of 1m, soil salinity (1.1-3.7 dS m^{-1}), water salinity (2.2 dS m^{-1}), pH of irrigation water (7.2), soil moisture at saturation (47%), and saturated hydraulic conductivity (Ksat= 300 mm/day) at the field site were measured or experimentally obtained in the Isfahan Soil and Water Laboratory. Table 1 shows some properties of the soil.

Description of the experimental treatments

The effects of various levels of consumptive water on GY and yield components of maize were determined using randomized complete blocks design as a split plot layout with three replicates and four treatments for 3 years (2000-2003). The levels of irrigation water which included control, 100%, 80%, and 60% level of water requirement were considered as the main plots and 2 varieties (single cross 704 and 647) as the sub-plots in the research station. The control was the conventional irrigation of the maize in the region. In the conventional irrigation treatment, the irrigation method, water amounts, timing and interval, as well as other irrigation criteria were considered based on the tradition of several years of local farmers. In this treatment, water losses during conveyance would usually result in lower irrigation efficiency. The irrigation amounts refers to the approximate historical average water applied by local farmers.

TABLE 1
Results of the soil samples analysis

Soil depth	EC	OC	P	K	N	FC	WP	BD	Texture
(cm)	(dS/m)	(%)	(ppm)	(ppm)	(%)	(m^3/m^3)	(m^3/m^3)	(g/cm^3)	
0-20	3.7	1.06	17.3	335	0.106	0.32	0.16	1.45	SiCl
20-40	1.2	0.78	5.1	250	0.078	0.36	0.17	1.42	SiCl
40-60	1.4	0.67	4.1	250	0.067	0.34	0.15	1.41	SiCl
60-80	1.1	0.15	2.3	260	0.051	0.35	0.16	1.43	CL
80-100	1.2	0.44	2.0	240	0.044	0.36	0.16	1.43	CL

EC, Electrical Conductivity; OC, Organic carbon; FC, field capacity; WP, wilting point; BD: Bulk density; SiCl, silty clay loam; CL, clay loam

The treatments were compared based on GY and yield components, which included 1000 Kernels Weight (1000 KW), kernel depth (KD), Kernel Number per Ear Row (KNER), and Kernel Number per Column (KNC) as means of GY improvement. GY was determined by grain weight for maize. This required a total of 24 plots (eight treatments with three replications). The maize was sown by hand at the end of May, at depths of 5-6 cm. The row spacing and crop distances on each row were 75 cm and 20 cm, respectively, giving a plant density of 90000 plant ha^{-1}. The length of each row was 30 m and there were four rows in each plot. The type and amount of the required fertilizers were determined from the analysis of the soil samples based on the instruction of the Soil and Water Research Institute (Malakouti, 1999). The N application used in this study was 500 kg ha^{-1} of N (urea at 46% N), which was divided into 3 applications (10 days before planting, 30 days after planting, every 15 days until 22 of July). The P (Amonium Phosphate) application added to the soil was 250 kg ha^{-1}. The K (potassium sulphate) application added to the soil was 350 kg ha^{-1}. Pests and weeds were controlled following the recommendations given by the Isfahan Pest Management Department. At harvest, the final total GY per plot was determined. At the end of September, the treatments were compared based on GY and yield components. The volume of water required for each treatment, taking into consideration an irrigation efficiency of 80%, was calculated based on the area cultivated and the depth of water. Irrigation intervals were seven days, which were based on the existing water rights in the region, and in accordance with irrigation scheduling. The different levels of irrigation water were applied based on the volumetric basis using siphons (2.54 cm diameter). The first irrigation by furrow irrigation method was implemented one day after seeding, with observed emergence about 6 days later. The source of water supply is an irrigation canal with EC equivalent to 1.2 dS m^{-1}. WP was calculated as the ratio of GY and the volume of applied water.

Irrigation Scheduling

The amount of evapotranspiration for irrigation scheduling was determined by using a crop coefficient (KC), ET_{pan} from measured daily open Class A pan evaporation data, and pan coefficient values from FAO 24 (Doorenbos & Pruitt, 1977). Irrigation water requirement was calculated as the difference between ETc (=KC times ET0) and the effective rainfall amount. In this study, pan evaporation and rainfall amount data collected from the Kabutarabad meteorological station located at the agricultural research centre were used for calculating irrigation water application quantities. The irrigation schedule was timed to meet the crop water requirement. The depth of irrigation water

and consequently the volume of water were applied weekly and irrigation amounts equalled the previous week's evapotranspiration (ETc) from the crop. Then, taking into consideration the discharge of the irrigation siphons, the relevant irrigation duration for each treatment was also determined. The data on the water requirement during the growing season for each period are shown in Table 2. Regardless of the difference between pan-evaporation and the evapotranspiration of vegetative surfaces, the use of pans to calculate ETo for the periods of 10 days or longer may be warranted (Doorenbos & Pruitt, 1977). Thus, to calculate ET0, 10-day periods were used in this study. The amounts of water to be used in the different irrigation level treatments were evaluated and these are shown in Table 3.

TABLE 2
Calculated irrigation water requirement of the maize in the region

	Period*	ET_c	ET_c	Net irrigation requirement
		(mm/day)	(mm/ Period)	(mm/ Period)
May	3rd	2.65	26.5	23.6
June	1st	2.82	31.0	29.0
	2nd	3.03	30.3	29.4
	3rd	3.76	37.6	37.6
July	1st	4.94	49.4	49.2
	2nd	6.24	62.4	61.8
	3rd	7.54	75.4	74.6
August	1st	7.82	86.0	85.5
	2nd	7.55	75.5	75.4
	3rd	7.36	73.6	73.6
September	1st	6.90	75.9	75.9
	2nd	6.29	62.9	62.9
	3rd	5.22	52.2	52.2
October	1st	3.77	37.3	37.6
	2nd	2.55	17.8	17.5
Total			794.3	785.8

* Each period is a 10 day in a month

TABLE 3
Amount of water (m^3 ha^{-1}) applied for different irrigation level treatments

Treatment/Month	Control	Irrigation level (100%)	Irrigation level (80%)	Irrigation level (60%)
June	1045	950	760	570
July	2769	2517	2014	1510
August	3357	3052	2442	1632
September	273	2482	1986	1489
October	935	850	680	510
Total	10836	9851	7881	5911

Statistical Analyses

The results were subjected to an ANOVA to analyze the effects of the treatments and their interactions. In order to determine the effects of year in different years of study, the data obtained were analyzed using the compound variance analysis and the averages of different treatments were separated using the Duncan multiple range test using the statistical software (SAS Institute, Inc., Cary, NC). The probability level of 0.05 (5%) was selected.

RESULTS AND DISCUSSION

Data Analysis

There is no interaction between irrigation and year, Irrigation × Variety, Variety × Year and Irrigation × Variety × Year (Table 4) for GY, 1000KW, KNER, KNC, and KD. The effect of irrigation levels on GY, KNC, and KD were found to be significant ($P \leq 0.05$) (Table 4). On the contrary, the effects of the irrigation level treatments on the 1000 KW and KNER were not significant. The effects of variety on GY and yield components were not significantly ($P \geq 0.05$) detected. The 60% irrigation level gave the lowest GY (8377 kg ha^{-1}) compared to the control, 100%, and 80% full irrigation (9250-9450 kg ha^{-1}) [Table 5]. KD and KNC at the 60% irrigation level were respectively reduced by 8% and 5.1% compared to the control treatments. The GY at the 60% irrigation level was reduced by 11.4% as compared the 100% treatments. The treatments of the control, 100%, 80% levels used for the GY, KD, and KNC were not significant. Thus, the 80% treatment can be utilized without any yield crop reduction when water is not limited, whereas the 60% treatment can be adopted when water availability is a limiting factor.

As indicated by the results, the 3-year average WP ranged between 0.83 and 1.42 kg m^{-3}. The WP was 0.835, 0.935, 1.167, and 1.422 kg m^{-3} respectively for the treatment control, 100, 80 and 60% levels of water use (*Fig. 2*). The highest WP was 1.43 kg m^{-3}, as calculated for the 60% treatment (means ± standard error). The effects of the control and full irrigation treatments on the WP were found to be higher than those under severe water stress such as the 60% treatment. On average, the increase in WP relative to that of the control treatment was 66% for the 60% treatment, 37.2% for the 80% treatment, and 12.8% for

TABLE 4
The variance analysis of different levels of irrigation on Maize GY, 1000 KW, KNER, KNC and KD indicators in the three years of the study period

Source of variation	df	GY	1000 KW	KNER	KNC	KD
Year	2	54.64^{**}	14131.92^{**}	15.71^{ns}	1287.27^{**}	31.8^{**}
Error (a)	6	0.87	462.17	5.09	22.36	0.44
Irrigation	3	0.91^{**}	298.34^{ns}	5.03^{ns}	24.14^{*}	4.99^{**}
Irrigation × Year	6	0.41^{ns}	243.99^{ns}	9.31^{ns}	16.09^{ns}	2.48^{**}
Error (b)	18	0.479	547.77	6.13	18.07	1.36
Variety	1	0.318^{ns}	33230.4^{ns}	256.74^{ns}	71.76^{ns}	22.680^{ns}
Irrigation × Variety	3	0.145^{ns}	496.44^{ns}	8.85^{ns}	2.266^{ns}	0.47^{ns}
Variety × Year	2	0.452^{ns}	146.03^{ns}	0.532^{ns}	5.07^{ns}	0.713^{ns}
Irrigation × Variety × Year	6	0.157^{ns}	214.67^{ns}	7.14^{ns}	5.99^{ns}	0.614^{ns}
Error (c)	24	0.359	248.29	5.4	6.97	0.505
%CV		9.8	5.2	13.5	6.3	5.9

** Significant at 1% level, * Significant at 5% level and ns Not significant

TABLE 5
The effects of different levels of irrigation on the quantitative indicators of the maize during the three years of research period (comparison of the mean of the treatments*)

Irrigation treatment	GY (kg/ha)	1000 KW (gr)	KNER -	KNC -	KD (mm)
Control	9271a	301.3a	16.7a	42.8a	12.4a
Irrigation level (100%)	9450a	308.8a	17.1a	43.1a	12.2a
Irrigation level (80%)	9250a	301.4a	18a	41.6a	12.6a
Irrigation level (60%)	8377b	299.8a	17.2a	40.6b	11.4b

* Comparison of the mean was done using Duncan test at 5% level. Mean with common letter is not statistically significant

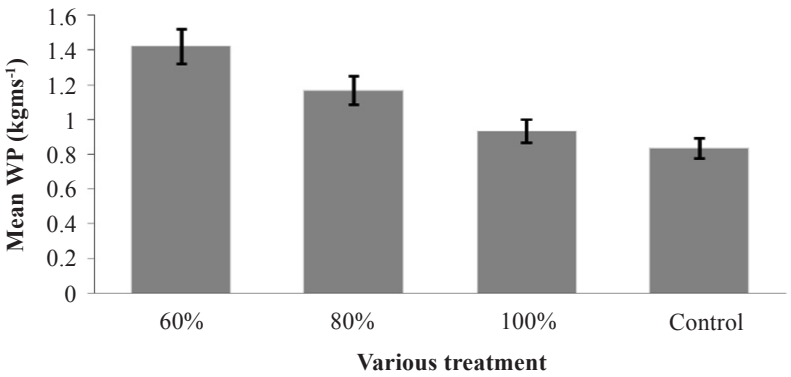

Fig. 2: Water productivity for different treatments (Bars represented standard error)

the full treatment cases, respectively. In *Fig. 3*, WP is plotted against irrigation water. This figure demonstrates how WP can be increased while simultaneously achieving water saving through reduced irrigations. WP was shown to be strongly affected by water deficit. The coefficient of determination of the regressed equation (R^2) index is 0.97, which shows high correlations between these two parameters for the three years of experimentation. Li *et al*. (2005) demonstrated that irrigation water use efficiency was negatively correlated with irrigated water volume.

Meanwhile, the regression analyses showed that the relationships between irrigated water volume and water use efficiency could be described by linear functions. Research conducted by Tavakoli (1999), Aghdaii & Sattar (2000), Cakir (2004), Payero *et al*. (2008) and Chen *et al*. (2009) revealed that the maize crop yield reduced with decreasing irrigation amounts, while maximum values of yield crop were obtained under fully irrigated treatments. Similarly, Oktem (2008) also found that water deficiency, together with hot and dry climate such as the GRB's summers, resulted in ear yield reduction. Most of the above stated studies have shown that maize yield is mostly affected by water stress. Payero *et al*. (2008), however, showed that the reported yield crop reduction for maize varied with location with differences in temperature and rainfall pattern, soil and crop characteristics, management practices, as well as weather conditions. However Kijne *et al*. (2003) believe that yield reduction is much lower than water reduction under the DI, whereas there is still potential for reducing crop water requirements to adopt more severe DI treatments and achieve the target of producing more crop yield per unit of water.

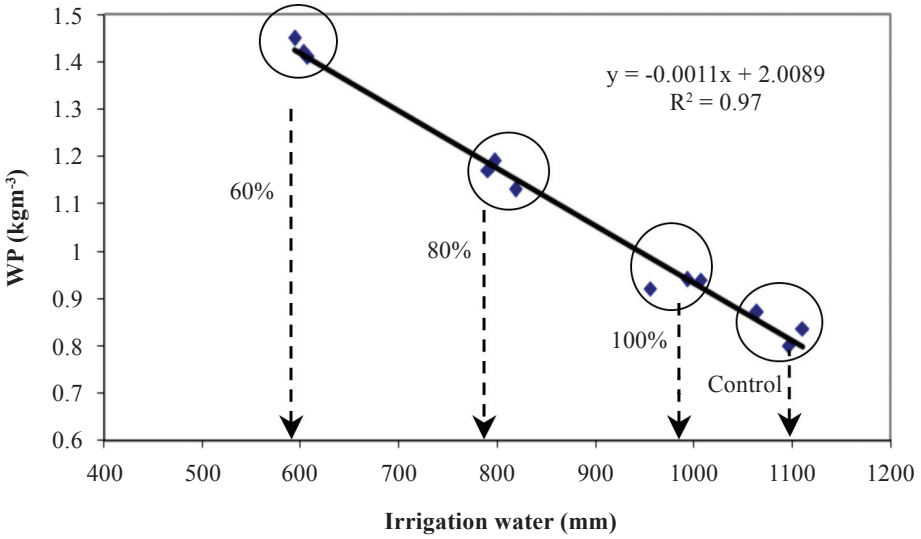

Fig. 3: The relationship between irrigation water and water productivity

Maize is the crop that is most sensitive to variations in plant density. The population for maize maximum economic GY varies from 30,000 to over 90,000 plant ha^{-1}, depending on water availability and nearly all environmental factors (Sangoi, 2001). Norwood (2000) and Emam & Ranjbar (2000) concluded that for maize, deficit-irrigation combined with proper fertility and plant population was a viable alternative to dryland in Kansas and Shiraz, where water resources are limited. In the present study, maize seeds were planted at the above plant density which might have resulted in a moderate reduction of maize yield under DI. Stone (2001) showed that water use efficiency (calculated the same as WP in this study) with water deficit increased as maize yield increased. The desire of most farmers is not to maximize WP, but to maximize profits. Therefore, there could be very good reasons for imposing deficit irrigation other than trying to maximize WP. Payero *et al*. (2006) demonstrated that trying to increase WP by applying deficit irrigation for maize might not be a beneficial strategy. Zwart & Bastiaanssen (2004) found that DI would probably increase WP only in situations where crops are being over-irrigated. The results suggested that if the crop was already deficitly irrigated, lowering irrigation inputs would only contribute to further reduction in yields and lower WP. Nonetheless, Oweis *et al*. (2004) have looked deeper into this issue and believe that there is a need to look for an optimum combination of production per hectare and production per m^3 volume of irrigation water to obtain "more food with less water". In other words, DI helps to stabilize crop yields and obtain maximum WP rather than maximum yields (Zhang *et al*., 2005). The above studies have also revealed that WP was strongly increased if crop water deficit was induced. These results are confirmed by the findings of the current study. A water deficiency level of 40% could therefore be acceptable for the maize (var. 704 and 647) in the central region of Iran and other similar arid and semi-arid ecological regions of the world.

CONCLUSION

During the three (3) years of the experiments with the four (4) irrigation treatments imposed on the crop, it was found that the grain yield and its components were mostly affected by the amount of irrigation water applied. The highest and lowest values of WP resulted from the treatment

60% level of water use and control, respectively. The highest magnitude of WP was calculated as 1.42 kg m^{-3} for the 60% treatment. Meanwhile, the general condition of the GRB is that there are many challenges involved in water shortage issues. Since demand for irrigation water far exceeded the supply in the years considered, the GRB succumbed to a severe deficit. In such a situation, the only way to keep supply and demand in balance is to reduce the allocation for agriculture. The good relationship obtained between WP and seasonal water consumption in this study can help and guide policy makers and planners come up with desirable solutions on how to manage water allocation for irrigated maize as the main summer crop in study area. This study has shown that with limiting water resources becoming a constant reality, deficit irrigation practices have become a priority for large irrigation networks. Considering the importance of water consumption optimization as the main scope in arid and semi-arid lands of Iran, a 60% level of water use is therefore highly recommended for agricultural maize production.

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Trans-scrotal Ultrasonography and Breeding Soundness Evaluation of Bulls in a Herd of Dairy and Beef Cattle with Poor Reproductive Performance

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ABSTRACT

The present study was undertaken to determine the fertility soundness of unselected bulls used for breeding based on a standard breeding soundness evaluation technique (BSE) and trans-scrotal ultrasonography (TSU). A total of 8 bulls, with the mean age of 5½ years (ranged from 3½ - 8 years) and the mean weight of 651.5kg (ranged from 480-840 kg) were evaluated. Three bulls were Friesian Sahiwal, 2 Brangus and the rest were Brahman-KK (Kedah Kelantan) cross, Simmental-KK cross and KK breed (one from each). Out of the total bulls examined, 3 (37.5%) were found to be unfit to be used for breeding due to their physical unsoundness and/or poor semen quality. The use of TSU has revealed the presence of testicular lesions in 3 bulls. The finding of TSU (suggestive of testicular degeneration) in one bull was reflected by a poor semen quality of the BSE results. Despite the presence of lesions of idiopathic unilateral hydrocele and bilateral fibrotic foci lesion in the other two bulls, the BSE findings for semen quality were not compromised. In conclusion, there was enough evidence to support our hypothesis that the observed drop in the reproductive performance of the herd was partly the result of using bulls with poor breeding soundness for fertility.

Keywords: Breeding soundness evaluation, bulls, poor reproductive performance, trans-scrotal ultrasonography

INTRODUCTION

Although artificial technologies for cattle breeding are rapidly improving and have progressively displaced natural service, as the preferred method of breeding in the dairy industries of most developed countries of the world, natural breeding is still the most common procedure used in beef cattle operations throughout the world. In U.S, for instance, more than 90% of beef cows are bred by natural service and each year, the use of bulls in natural mating programmes accounts for over 95% of pregnancies (Kreplin, 1992; Perry, 2008). Where natural breeding is employed, reproductive statistics on the farm is greatly influenced by the fertility and handling of the bulls. To impregnate a cow in a natural mating situation, the bull must produce semen of satisfactory quality and be able to successfully mount and deposit the semen in the reproductive system of the cow. Failure to meet either criterion will result in poor reproductive performance (Kreplin, 1992).

Studies done on the prevalence of bulls unfit for use in breeding programmes showed that

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approximately 1 in every 5 bulls had inadequate semen quality, physical soundness, or both (Barth & Waldner, 2002). Furthermore, some survey studies have indicated infertility as the most important reason considered when culling a bull (Parkinson, 2004). Moreover, unlike a cow which is responsible for half of the genetic makeup of one calf per year, the bull which is responsible for half of the genetic makeup of a number of calves per year depends on the bull to cow service ratio. This makes determining the potential fertility of the bull much more important than determining the fertility of any individual cow. Hence, the role of the bull in the fertility of both beef and dairy cows needs to be understood, particularly in terms of the overall constraints to cattle fertility (Kreplin, 1992; Chenoweth, 1999; Parkinson, 2004).

Breeding soundness evaluation (BSE) of bulls has been extensively used for evaluation of male fertility prior to the breeding period over the past 50 years (Hoflack *et al*., 2008). BSE is a useful tool in identifying bulls with reduced fertility or physical problems which lower their ability to sire calves. Thus, eliminating the bulls with physical problems or reduced fertility from the breeding herd improves the overall reproductive efficiency of the herd (Bagley & Chapman, 2005).

According to the Society for Theriogenology (SFT), bull BSE comprises general and reproductive physical examination, scrotal circumference indexed for age, semen motility and sperm morphology examinations (Alexander, 2008). Additional parameters, such as trans-scrotal ultrasonography, have been reported recently to enhance the routine BSE in bulls (Chapwanya *et al*., 2008). Trans-scrotal scanning is a convenient, non-invasive technique which allows an examination of both palpable and non-palpable testicular lesions in goats and rams (Ahmad & Noakes, 1995), making its use ideal in farm situations (Chapwanya *et al*., 2008). However, the application and interpretation of its findings in determining fertility status of the bulls in relation to the routine BSE technique still require further research.

Despite the potential effect of the bulls' fertility on the overall reproductive efficiency of cattle herd, their role in the breeding programmes is often underestimated and the use of standard BSE for bulls before use for breeding has been overlooked. This allows poor performing bulls to be used for breeding and hence leads to reduction in an overall reproductive performance of the herd. The farm at Universiti Putra Malaysia, where this study was conducted, follows a similar extensive management system, whereby the bulls of unknown fertility status have been used without prior detail BSE. A drop in the overall reproductive performance of the cattle in the farm was observed with a number of beef and dairy cows with prolonged post-partum period and heifers with delayed age at first calving (Yimer *et al*., 2010). Our previous study (Yimer *et al*., 2010) has shown a considerable number of females having normal ovarian activity but failed to conceive. Thus, it was hypothesized that these animals are unable to become pregnant due to bulls' fertility-related factors. As a result, this study was designed to investigate the soundness of breeding bulls for fertility in a dairy and beef cattle herd.

MATERIALS AND METHODS

Animals and Management

A total of 8 bulls with the average age of 5½ yrs (ranged from 3½ - 8 years), and with a mean body weight of 651.5 kg were used for natural mating on the farm in this study. Three of the bulls were Friesian Sahiwal, which were used to serve dairy cows of the same breed. Meanwhile, the remaining 5 bulls consisted of one KK, a Simmental-KK cross, a Brahman-KK cross, and 2 Brangus bulls. The 5 bulls were used to serve the beef females of KK cross, Brangus and Bradford crossbreeds.

The bulls were mixed with the females for breeding at a service ratio of 1 to 30. A bull will remained with the females for breeding for about 2 to 3 weeks before it was replaced by another. During the resting period, the bulls were kept in stalls with shades. Feeding was mainly

based on palm kernel cake (PKC), daily supply of 5 kg/animal, in combination with mineral supplement. Fresh grass supplement and water were provided *ad libitum*. As part of the herd's health management, all the bulls were vaccinated against foot and mouth disease and haemorrhagic septicaemia. These bulls were also diagnosed to be negative for diseases, such as vibriosis and brucellosis.

Breeding Soundness Evaluation

Medical history and physical examination

A standard BSE technique, developed by the Society for Theriogenology SFT (Hopkin & Spitzer, 1997) was used in this study. Prior to semen collection, the identification of each bull, as well as the medical and breeding histories, were recorded. The general and detail physical clinical examinations were performed on each bull, following the procedure described by Alexander (2008). Moreover, information related to the evidence of pain during mounting was also recorded. General clinical examination was made by giving attention to the condition of teeth and jaws, eyes, thorax, abdomen, feet, and the legs. A detail physical examination of the reproductive organs was performed to evaluate the scrotum for abnormalities and its circumference, testicles (consistency and asymmetry), penis and prepuce for any defects, and all accessory sex glands palpated per rectum for abnormalities.

Semen collection

A total of 32 semen collections (4 from each bull on average) were made at 2 to 3 weeks interval. Semen samples which were contaminated with urine were not used for the evaluation. As the bulls were not trained for artificial vagina, electroejaculation (EE) was used to collect semen for evaluation. In some bulls, rectal massaging at the area of ampulla was applied to get semen when they failed to respond to EE. An electro-ejaculator (The P-T electronics, model 304, Oregon, USA), fixed to a probe measuring 3 cm in diameter with 3 longitudinal electrodes, was used to stimulate ejaculation. After placing the probe in the rectum with electrodes facing down, a voltage range (0-15 volt, depending on the response of the animal) was used with gradual increase to the desired stimulus, holding for 2-3 seconds and then decreased to zero over a 2-3 second span. After 2-3 seconds in the off position, the cycle was repeated. Prior to semen collection, hair around the prepuce was removed and the area of the prepuce was cleaned thoroughly with water and dried using clean paper towels to minimize contamination. Immediately after the semen was obtained using clean graduated test tube attached to a rubber cone, records were taken for colour and volume; the semen was then kept in warm water (37°C) and transported to the laboratory (Faculty of Veterinary Medicine, Universiti Putra Malaysia) for further analysis within 20-30 minutes.

Semen analysis

A conventional method was used to analyse sperm morphology using eosin nigrosin stain, which was dried in air and examined under light microscope, 1000x magnification (Goovaerts *et al*., 2006). A drop of net semen was mixed to 4 drops of eosin nigrosin stain for 30 seconds and a smear was prepared by taking 5µl of the mixed sample. A total of 200 spermatozoa was examined in each sample, and expressed as percentages of the normal spermatozoa and spermatozoa with abnormal morphology, such as head, mid-piece and tail abnormalities, and spermatozoa with a proximal or distal protoplasmic droplet.

In addition to the conventional method, Computer Assisted Semen Analysis (CASA) technique, which involves a computer aided semen analyser with animal motility software (IVOS 12.2, Hamiltone Thorne Biosciences, USA), was used to objectively determine the concentration, general, and progressive motility of spermatozoa.

Depending on the density of the ejaculate, the sample was diluted in normal saline (1:100 or 1:50). From the diluted sample, a 10µl

aliquot was loaded on a 2X-CEL, 20µm dual side sperm analysis chamber (Hamiltone Thorne Research, USA) and inserted into the computer for analysis at a stage temperature of about 37°C. A total of 8 fields (4 fields from each chamber) was randomly analyzed and their results were averaged and used to compare with the standards. Based on the guideline by SFT, minima of 30% progressive motility, 70% normal morphology and age-related scrotal circumference (minimum 34 cm for age >2 yrs) were used as standards. Bull set up number 4 of the CASA software with slight adjustment on some parameters, based on the preliminary trials for the exact identification of sperm cells using the play back facility, was used for the analysis. The parameters set during the analysis are presented in Table 1 below.

Trans-scrotal ultrasonography

A B-mode, real time portable ultrasound scanner with a 5 MHz linear array transducer (SSD 555, Aloka, Japan) was used for the trans-scrotal ultrasonography along the mid-sagital and transverse plane of each testis. An ultrasound gel was applied to the face of the transducer to maintain a proper contact and to avoid interference of air between the surface of the transducer and the testis.

Statistical analysis

In this study, descriptive statistics was used to express the mean and percentage of each semen quality parameter, whereas motility and morphology were computed using SPSS version 16.

RESULTS

Evaluations of Breeding Soundness

A summary of the results from the BSE for each bull is given in Table 2. Out of the total stud bulls examined, 37.5% (3/8) of them failed to meet the standards for BSE and found to be unfit

TABLE 1
Software settings of the Hamilton Thorne IVOS 12.2 used in the present study for the bull sperm motility assessment (Farrell *et al*., 1998)

Parameter	Parameter function	Value
Frame rate (Hz)	Image capture	60
Number of frame required	Image capture	30
Minimum contrast	Cell detection	60
Minimum cell size (pixels)	Cell detection	4
Non-motile head size (pixels)	Cell detection	5
Non-motile head intensity	Cell detection	60
Medium VAP cut-off (µm/s, MVV)	Progressive cell detection	50
Straightness cut-off (%, STR)	Progressive cell detection	70
Low VAP cut-off (µm/s, LVV)	Static cell detection	30
Low VSL cut-off (µm/s, LVS)	Static cell detection	15
Minimum static intensity limit	Cell differentiation	0.3
Maximum static intensity limit	Cell differentiation	1.7
Minimum static size limit	Cell differentiation	0.56
Maximum static size limit	Cell differentiation	4.74
Minimum static elongation limit	Cell differentiation	0
Maximum static elongation limit	Cell differentiation	85

for breeding, due to either physical unsoundness and/or poor semen quality. One bull (ID T025) failed BSE because of the left hind limb lameness, which has existed for a year before the evaluation. The lameness was pronounced during mounting. For this bull, however, other parameters related to semen quality and scrotal circumference were above the minimum standards set by the SFT. The mean values for general sperm motility, progressive motility, and spermatozoa with normal morphology were 63.6%, 41.5%, and 74.5%, respectively, with 43 cm scrotal circumference (Table 2).

The second bull (ID T036) showed unsatisfactory semen quality for the percentage of normal sperm morphology (66.9%), which was less than the minimum standard (70%). The semen from this bull contained 63.8% general sperm motility and 43.7% progressive motility. This bull had the smallest scrotal circumference (35cm) compared with the other bulls, but this was slightly above the minimum standard set by SFT (Table 2).

Right testicular degeneration and poor semen quality were the findings in the third bull (ID 3770, Brangus breed). The right testicle appeared to be atrophied or degenerated as its size was about 1/3 from the left testicle. The semen quality analysis showed all the parameters far below the minimum standards; 63.4% mean sperm cells with normal morphology, progressive motility (19.8%) and asymmetrical testicles. The mean value for sperm concentration (425 X10^6/ml) from this bull was also the lowest compared with the other bulls examined (Table 2). Meanwhile, the medical history of the bull indicated the presence of inflammatory swelling of the right testicle previously, which later recovered following antibiotic treatment.

Findings of Trans-scrotal Ultrasonography

The use of trans-scrotal ultrasonography has revealed the presence of lesions in the testes of three bulls. One of them (ID 3770) had right testicular atrophy that failed to meet the minimum standards for BSE. The ultrasonographic examination of the right testicle of this bull (*Fig. 1a*) showed the presence of non-homogeneous, hyperechoic fibrotic testicular parenchyma with the size reduced to about 1.6cm (as measured from the mediastinum to the border of the testis) compared with the normal left testicle which had a medium to

TABLE 2
Summarized data obtained from breeding bulls based on BSE

ID	Breed	Age (yrs)	SC (cm)	Physical soundness	Average semen quality parameters from CASA/ conventional method (morphology)			
					Concen-tration (M/ml)	General motility (%)	Progressive motility (%)	Abnormal morphology (%)
T025	Fr.Sahiwal	5	43	Lameness of left limb*	546	62	37	21
T036	Fr.Sahiwal	3 ½	35	Sound	697	64	44	33.1*
1545	Fr.Sahiwal	7	46	Sound	1044	76	43	21.6
2519	Simm.KKx	6 ½	36	Sound	1042	67	47	15
B505	Brangus	3 ½	44	Sound	693	68	51	27.6
3770	Brangus	8	NA	Right-testicular degeneration*	425	38	20	36.6*
3549	KK	5 ½	36	Sound	1250	65	64	20.6
3568	Brah.KKx	5	43	Sound	827	62	43	22.0

NA: not applicable; KK: Kedah-Kelantan; Fr: Friesian; Brah: Brahman; Simm: Simmental; SC: Scrotal Circumference; *parameters below the minimum standard
Minimum standards: 30% progressive motility; 70% normal morphology (< 30% abnormal morphology); 34cm SC for age ≥ 2yrs

coarse echogenicity (*Fig. 1b*). A unilateral anechoic fluid-filled space (hydrocoele) between the parietal and visceral layers of the vaginal tunic which measured about 1.6cm in the right testicle of bull 3568 was the other lesion detected with ultrasonography (*Figs. 2b* and *2c*). However, ultrasonogram of the left testicle of the same bull (*Fig. 2a*) and also testes of the other bulls examined did not show such anechoic space. The third lesion, which involved both testicles, was noted in bull with ID 1545. The ultrasonogram of the testes showed the presence of hyperechoic foci scattered throughout the testicular parenchyma with acoustic shadowing, consistent with fibrosis and possible mineralization (*Fig. 3b*).

Despite the presence of lesions mentioned in the bulls, ID 3568 and 1545, semen quality parameters, such as motility and morphology, were not affected. Moreover, ultrasonography of the testes of a bull (ID T036) that failed BSE because of the high percentage of abnormal sperm morphology (*Fig. 4*) and other bulls did not show any abnormal lesion.

DISCUSSION

Kastelic & Thundathil (2008) showed that 20 – 40% of bulls had reduced fertility in an unselected population for fertility soundness. Moreover, a 33% prevalence of the bulls unfit for breeding had also been reported by Chacon *et al*. (1999) from extensively managed bulls in Costa Rica based on BSE. Eliminating such bulls with physical problems or reduced fertility from the breeding herd improved the overall reproductive efficiency of the herd (Bagley & Chapman, 2005). In the present study, 37.5% (3/8) of the stud bulls, which have been used for breeding in the farm, were found to be unfit for breeding due to either physical problems or inadequate semen quality, or both. The occurrence of subfertile bulls indicates their role in the reduction in the overall fertility of the herd. All these subfertile bulls were eventually culled from the breeding group.

During the mating act, the full weight of the bull is borne on the hind legs and feet, therefore, any unsoundness in this area drastically interferes with its breeding ability (Barth, 2007). A bull with physical problems of the legs, eyes, testicles, penis, or internal

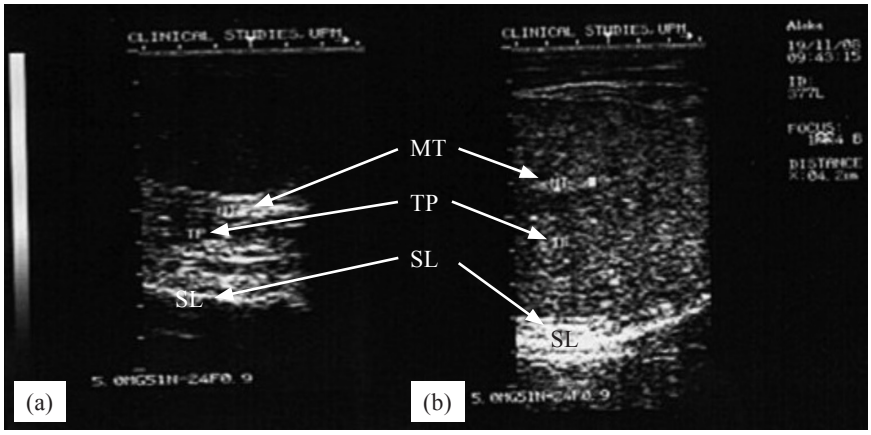

Fig. 1: Testicular ultrasonography of a Brangus bull (ID 3770); a) ultrasonogram of the right testis undergoing degeneration/atrophy with hyperechogenic, fibrotic and diminished sized testicular parenchyma (TP), and b) homogeneously granular; normal appearance of the left testicular parenchyma. The size of TP, as measured from mediastinum testis (MT) to border of testis near the scrotal layer (SL), was 4.2 cm while 2.6 cm for the right testis

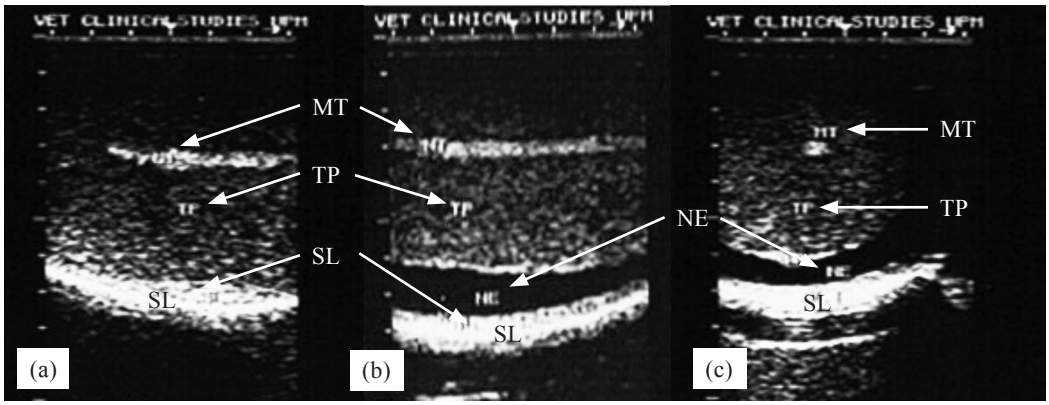

Fig. 2: Testicular ultrasonography of a Brahman-KK cross bull (ID. 3568); a) normal ultrasonogram of the left testicle scanned along the mid-saggital plane with no space between the testicular parenchyma (TP) and scrotal layer (SL), b) right testicular ultrasonogram with completely dark nonechoic (NE) fluid filled space (hydrocele) between the border of the testis and the scrotal layer (SL) measuring 1.6 cm, and c) shows the same right testis when scanned along the transverse plane with the hydrocele (NE) indicated by the arrow

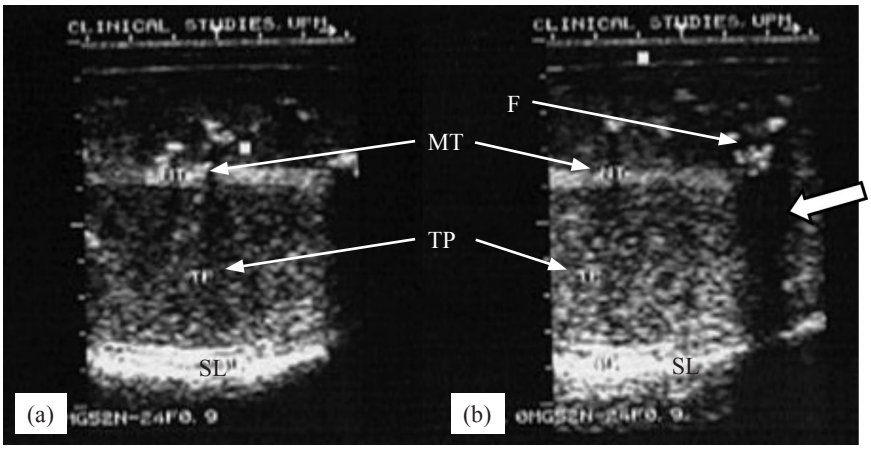

Fig. 3: Testicular ultrasonography of a Friesian Sahiwal bull (ID. 1545). Ultrasonogram of both the left (a) and the right (b) testes with hyperechogenic foci (F) scattered throughout the parenchyma forming acoustic shadow beneath the foci as indicated by the biggest arrow in (b)

reproductive structures that interfere with its ability to impregnate females is unfit for breeding, as long as the problems are unlikely to improve with time (Bagley & Chapman, 2005). In the present study, two of the bulls examined showed physical problems related to the leg (hind limb lameness in bull T025) and the testis (testicular atrophy in bull 3770). According to the medical history, both problems had chronically existed for about a year prior to the evaluation, indicating that these problems were unlikely to recover with time. In addition to the

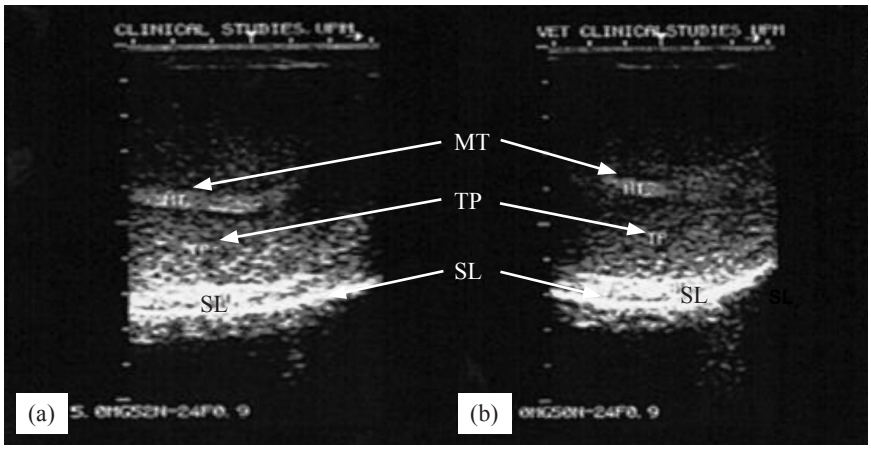

Fig. 4: Testicular ultrasonography of a Friesian Sahiwal bull (ID. T036). The ultrasonogram of both the right (a) and the left (b) testes show normal granular and homogenous testicular parenchyma (TP), with relatively more echogenic mediastinum testis (MT) and scrotal layer (SL). The small sized scrotal circumference (35 cm) is reflected by the presence of small sized parenchyma portion as viewed in the ultrasonogram compared with other bulls examined

unilateral testicular atrophy observed in bull 3770, the quality of semen was demonstrated by low percentage of progressive sperm motility (20%) and normal sperm morphology (63.3%), which were both below the minimum standards set by SFT. The poor semen quality is likely due to the degeneration of the left testicle. Testicular degeneration is an acquired condition in which one or both testicles that were once normal undergo pathologic changes consequently, resulting in small testicular size and abnormal function (Hopkins, 2007). It has been reported that degenerative changes in the testicle are frequent causes of infertility in males, including bulls which commonly occur following inflammatory reaction to infection (Barham & Pennington, 2006). Testicular degeneration results in testicular dysfunction which is responsible for poor semen quality (Hoflack *et al*., 2008). The swelling associated with inflammation, coupled with an inelastic tunica albuginea, tends to cause pressure necrosis of the testicles. Thrombosis of blood vessels results in degeneration which causes the testicle to lose its ability to function normally. In addition, the increased temperature of the scrotal contents has been shown to result in testicular degeneration with a higher percentage of abnormal ejaculated sperm (Hopkins, 2007).

Several researchers have shown that there is a positive relationship between scrotal circumference, male reproductive traits and semen quality parameters, such as the number of normal sperm, sperm concentration, sperm motility, and total daily sperm production (Madrid *et al*., 1988; Geske *et al*., 1994; Chacon *et al*., 1999). Consequently, many reports have demonstrated that scrotal circumference is positively related to conception and/or pregnancy rates (Coulter & Kozub, 1989; McCosker *et al*., 1989; Makarechian & Farid, 1985; Larsen *et al*., 1990; Brito *et al*., 2002; Fitzpatrick *et al*., 2002; Holroyd *et al*., 2002). In addition, selecting bulls for large testes has been reported to improve the fertility of their female offspring by reducing the age at puberty and breeding (Barham & Pennington, 2006). One of the bulls evaluated in this study (ID T036) had high abnormal sperm morphology (33.1%), beyond the minimum standard (30%). No clinical abnormalities were apparent upon palpation of the testes and ultrasonography. However, this bull had the

lowest scrotal circumference (35 cm, age 3½ yrs) compared with the rest of the bulls. This value is slightly higher than the minimum standard set by SFT for fertility soundness (minimum of 34 cm for age ≥ 2 yrs). It has shown a 1cm increment only in its additional 1½ yr life time. Being the youngest of all the bulls examined, the age may have played a role for the high percentage of abnormal sperm morphology obtained.

The use of trans-scrotal ultrasonography in the assessment of bull's soundness for fertility was evaluated in this study. Out of the total eight bulls examined, the ultrasonography of the testes revealed the presence of lesions in three bulls. Unilateral testicular degeneration in bull 3770 was one of the lesions found. Meanwhile, the ultrasonogram of this testicle showed the presence of a hyperechogenic fibrotic tissue with diminished size and loss of homogeneity in the parenchyma, compared with the normal contralateral testicle. This finding of ultrasonography enhanced the fertility assessment of bull 3770 which was found to be unfit for breeding, based on the routine BSE procedure. Ultrasonography has given a more detailed explanation for the poor quality of semen obtained from bull 3770 which was likely due to the increased fibrotic tissue in the parenchyma of the affected testis. This is in accordance with Chapwanya *et al*. (2008) who have reported that there is an agreement between trans-scrotal ultrasonography and BSE in classifying a bull's fertility. It has been described that a decrease in the sperm concentration and motility, in combination with an increase in morphological abnormalities demonstrated in several Belgian Blue bulls, is associated with testicular degeneration (Hoflack *et al*., 2008). Moreover, Hahn *et al*. (1999), who compared ultrasonogram of Holstein bulls testes with high semen quality and bulls with low semen quality using low frequency transducers (1.6 and 2.25 MHz), discovered the presence of substantial fibrotic tissue (more parenchyma tissue replaced with connective tissues) in the testes of bulls with poor quality semen.

The other two testicular lesions revealed in the current study were idiopathic scrotal hydrocele (seen as unilateral fluid filled anechoic space in the vaginal cavity on ultrasound) and bilateral scattered fibrotic foci found in a Brahman-KK cross (3568) and Friesian-Sahiwal (1545) bulls, respectively. Despite the lesions found in the two bulls, BSE showed a sound fertility status with satisfactory semen quality and scrotal circumference above the minimum standard. The larger the testicular size in both bulls (3568 & 1545) and the unilateral nature of the hydrocele found in bull 1545, thought to have played a role for the quality of semen not to be compromised. A hydrocele is an abnormal accumulation of serous fluid in the vaginal cavity (the space between the parietal and visceral layer of tunica vaginalis layer found immediately covering the testes) (Bartholomew *et al*., 2003). In normal bulls, the parietal and visceral layers of the vaginal cavity were not observed separately as examined by ultrasonography. However, a space that does not exceed 2 mm width has also been reported as normal (Pechman & Eilts, 1987). Temperature induced dysfunction of spermatogenesis is generally thought to occur because of the insulating effects of the fluid in a hydrocele. Consequently, fertility can be guarded especially in cases where bilateral hydroceles persist (Schumacher & Varner, 2007). Brito *et al*. (2003) reported whole scrotal insulation resulted in decreased sperm production and semen quality in *B. indicus* and *B. indicus* x *B. taurus* bulls without changes in testicular echo-texture. Similarly in this study, there was no change in the echo texture in the testicle of the bull with hydrocele. Shore *et al*. (1995), who did a follow up study on the scrotal sonogram of hydrocele and semen quality, reported spontaneous resolution of hydrocele within 120 days in 85% of bulls (22/26). The percentage of bulls with unilateral hydrocele resolved was higher than those with bilateral hydrocele (94 vs. 62.5). The researchers also reported that at 90 days, semen quality was satisfactory in most bulls with unilateral hydrocele which was higher than those with bilateral problem. The hydrocele observed in this study was reduced significantly after 2 months' period.

Meanwhile, the lack of correlation between the findings of multifocal echodensities in the testicular parenchyma and poor breeding soundness score or with a high number of spermatozoa abnormalities have been reported by Shore *et al*. (1995). Similarly in this study, BSE showed an acceptable fertility status with no adverse effect of the foci lesion on semen quality despite the scattered foci lesion in one of the bulls examined (1545). Nevertheless, racket-shaped sperm head abnormal morphology, which was frequently examined in bull- 1545 (data not shown), might be associated with the scattered hyperechoic foci lesion found in the testes. In general, the lesions revealed by ultrasonography in this study were not detected through palpation in a routine BSE. This shows the significance of trans-scrotal ultrasonography in the assessment of bulls for the soundness of fertility although semen quality is dependent on the severity of the lesions and the overall scrotal/testicular size of the bull.

CONCLUSION

The current study clearly showed the consequence of underestimating BSE for breeding bulls of unknown fertility status which is demonstrated by the occurrence of 3/8 bulls with unsound fertility that lead to the drop in the overall reproductive efficiency of the herd. Moreover, the study enabled to evaluate the use of TSU in the assessment of bull's soundness for fertility. TSU using a 5MHz transducer was found to be a useful non invasive and easily applicable technique in assessing the health status of the testicle in detail, revealing lesions which were difficult to be detected by palpation, consequently enhancing the routine BSE technique. Despite the presence of the lesions in the testes of the two bulls (bull 1545 and 3568), their semen quality was not affected and BSE result showed a sound fertility status. The authors are of the opinion that the larger size of scrotal circumference (43 and 46 cm) or testicular size, as a compensatory factor in both bulls and the unilateral existence of the hydrocele in bull 3568, have played a role for the semen quality (normal morphology and motility) not to be affected compared to the standards. However, there is a need of further research to estimate the extent of lesion obtained by US that can produce substantial effect on the quality of semen below the standard set by SFT. In this study, it was generally noted that ultrasonographic findings of a unilateral hydrocele (width, 1.6 cm and scrotal circumference, 43 cm) in a Brahman-KK cross bull and bilateral presence of scattered fibrotic foci with acoustic shadow (SC, 46 cm) in a Friesian Sahiwal bull have no significant effect on the quality of semen and soundness for fertility.

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Relationships of Distribution of Macrobenthic Invertebrates and the Physico-chemical Parameters from Semenyih River by Using Correlation and Multiple Linear Stepwise Regression Analyses

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ABSTRACT

The distribution of macrobenthic invertebrates at Semenyih River has been described by Yap *et al.* (2003a), but their relationships with physico-chemical characteristics of the river have yet to be established. By using correlation and multiple linear stepwise regression, it was found that BOD_3, orthophosphate, total suspended solids and turbidity were important in structuring the stream macrobenthic invertebrate communities because they determined whether organisms could colonize and persist in the stream habitats. Thus, the invertebrates are useful as bioindicators to the health of the river ecosystem, complementing water quality analysis. Impacts of anthropogenic inputs can therefore be assessed based on the macrobenthic invertebrates' different species distribution.

Keywords: Semenyih River, macrobenthic invertebrates, correlation analysis, multiple stepwise regression analysis

INTRODUCTION

A previous study reported by Yap *et al.* (2003a) at Semenyih River on a list of the macrobenthic invertebrates poses, among the non-biologists, a question on 'How valid is the bioindication concept be employed in the river pollution study?' In order to answer this question, the relationships between the distribution of macrobenthic invertebrates and the physico-chemical parameters of the river need to be conducted. Meanwhile, the analyses of the water quality and macrobenthic invertebrates has their respective advantages. For instance, water quality could give a rapid assessment on the water quality status of the river, while bioindication concept could reveal the health of the river ecosystem in response to pollution (Mason & Parr, 2003; Yap *et al.*, 2003a; Azrina *et al*., 2006).

Regardless of which methodology (water quality or bioindicators) is employed, the relationships between water quality of the sampling sites and the distribution of macrobenthic invertebrates should be informative of the environmental quality of the river ecosystem, since in fact, both the physical-chemicals and biotic information/ methodologies are giving the actual picture of the quality of the river being studied (Azrina *et al.*, 2006). Since ecological studies on the macrobenthic invertebrates involving their abundance and distribution are interrelated with their surrounding, such as physico-chemical components in which they are found at the

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bottom of the rivers, the relationships between the abundance/distribution of the macrobenthic invertebrates and the physico-chemical parameters of the habitat river waters should be conducted. Among the multivariate statistical analyses, correlation and regression analyses are among the most common methods used to show the relationships between the organisms and their surrounding since they have been widely used as reported in the literature (Yap *et al.*, 2010). In addition, Yap *et al.* (2003b) also used multiple linear stepwise regression (MLSR) in the study conducted on heavy metal accumulation by the green-lipped mussel, *Perna viridis*.

According to Norris *et al*. (1982), contaminants that may have impacts on aquatic system should be assessed by an interpretative study of the physico-chemical characteristics in relation to the biota. A biodiversity of the benthic communities, being dependent on the conditions and resources of its locations, may change if environmental factors change. Meanwhile, due to the complex interrelationships and the many environmental factors of the natural river ecosystem, only certain influential factors could potentially affect the distribution and abundance of macrobenthic invertebrates. An alteration in these conditions or resources that lead to one or more of these conditions to change may cause many of the populations to change, and be replaced by others (Warren, 1971). Therefore, the use of MLSRA is more logical to test and find out the most influential parameter that affects the density of macrobenthic invertebrate in Semenyir River. The objective of this study was to determine the relationships between the physico-chemical parameters and distribution of macrobenthic invertebrates found at Semenyih River using the correlation analysis and MLSRA.

MATERIALS AND METHODS

Study Area

This study covered the riverine system of Semenyih River, (2° 54'N to 3° N and 101° 48'E to 101° 53'E), a tributary of the Langat River. The sampling was conducted in June 1997. Seven stations were established along the river, with St-1 being the closest to Semenyih Dam (most upstream) and St-7 farthest to the downstream (*Fig. 1*). The sampling technique used for the macrobenthic invertebrates, their preservation and identification at the sampling sites and the list of macrobenthic invertebrates found in Semenyih River have already been reported by Yap *et al*. (2003a).

Water Quality Measurement

The physico-chemical characteristics of the stream, recorded directly at each sampling site, were water velocity, temperature, depth, river width, conductivity, pH and dissolved oxygen. Water velocity was measured by direct timing (Stopwatch, string and ping pong ball), whereas river depth and width were done using a meter ruler and measuring tape, respectively. The conductivity, temperature, and dissolved oxygen were measured using the YSI S-C-T meter, while pH was determined using a pH meter, i.e. Orion 410A+.

Samples of water were stored in polyethylene bottles (500 ml). Orthophosphate, nitrate, ammonia, turbidity and total suspended solids (TSS) were in accordance with the Standard Methods (APHA, 1985). Water in polyethylene bottles were preserved with 2 ml of concentrated hydrochloric acid (pH < 2.0) and brought back to the laboratory. These water samples were cleared by any suspended solids with paper filtration, except for the determination of turbidity. Meanwhile, the concentrations of nitrate, orthophosphate, ammonia and turbidity were determined using the spectrophotometer model HACH DR 2000 with specified wavelengths of 507 nm, 890 nm, 425 nm, and 450 nm, respectively. Blanks used to obtain the zero values were from 25.0 ml of deionised water that were put into the other sample cells.

For Biochemical Oxygen Demand (BOD_3), Wheaton bottles (300 ml) were filled until overflowed so that there were no bubbles inside the bottles before topping them with stoppers. These bottles were then put inside a cool box as

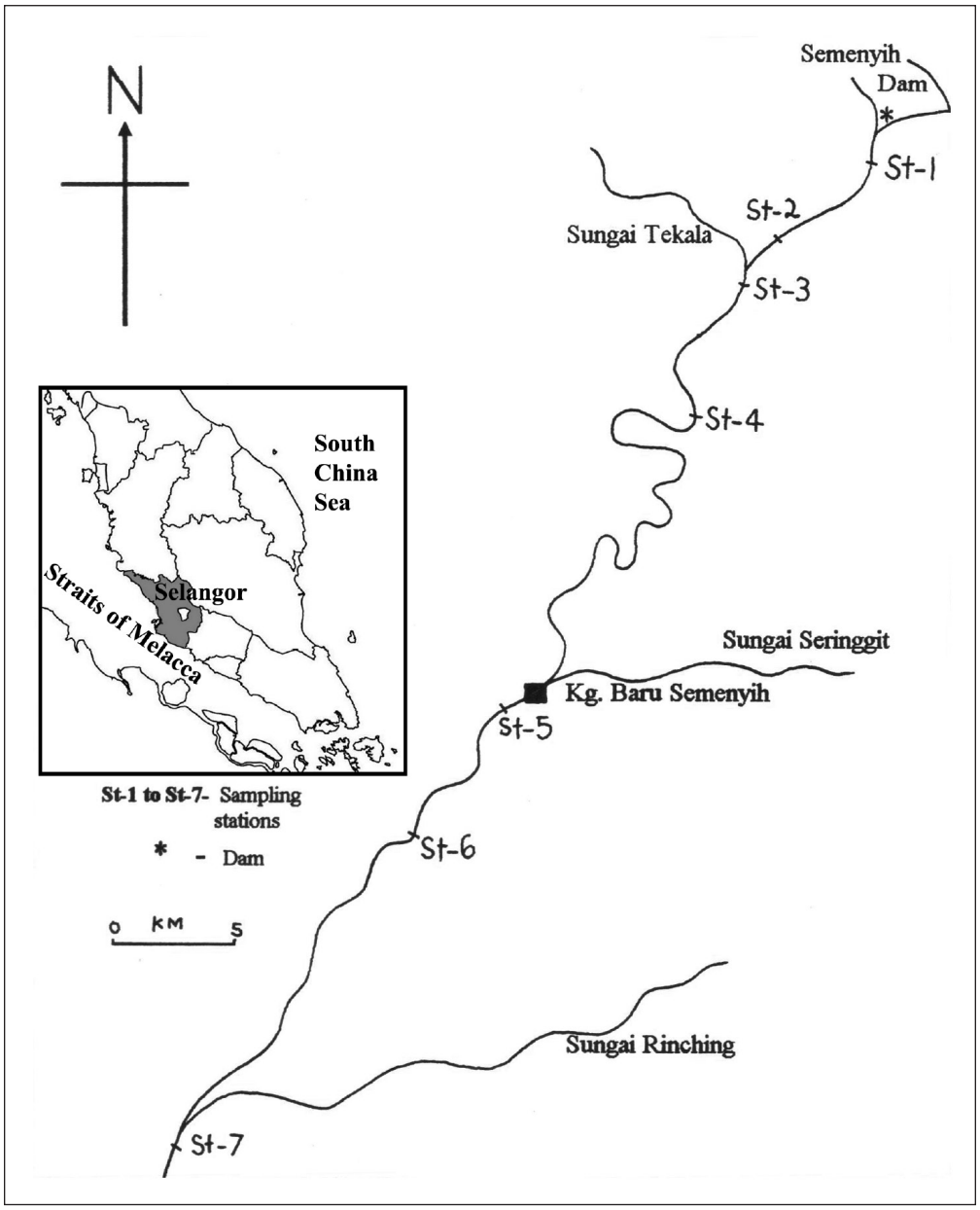

Fig. 1: The locations of the sampling stations in this study (The Directorate of National Mapping, Malaysia, 1982)

soon as they were collected in the field. At the laboratory, the initial DO was measured before 3-days incubation at room temperature (28ºC).

Statistical Analyses

The data transformation was carried out to normalize the data and reduce the errors. All the converted physico-chemical data were transformed by log10 while the macrobenthic

invertebrates' density data by applying fourth root. Pearson's correlation analysis and MLSRA were carried out on the transformed data using the Statistical Analysis System Version 6.0 (SAS Institute, 1987).

RESULTS AND DISCUSSION

Physico-chemical Parameters of the River

The overall physical and chemical characteristics of Semenyih River are given in Table 1. In general, river width, total suspended solids, turbidity, BOD_3, orthophosphate, nitrate, and conductivity increased with the increasing distance from Semenyih Dam. The overall river widths of the region ranged from 5.58 (St-2) to 32.67 m (St-7). In regard to the total suspended solids and turbidity, both ranged from 5 to 147 mg/L and from 2.33 to 122.00 FTU, respectively. The highest TSS and turbidity recorded downstream (St-7) was due to the instability of the river bottoms (Coker, 1968) that was caused by land-based activities, including erosion and addition of human wastes. The significance of the suspended solids in natural water normally relates to their influence on light and sediments (Maitland, 1990). As for the BOD_3, it was found to have increased from 0.60 (St-1) to 3.37 mg/L (St-7). The concentrations of nitrate ranged from 0.10 (St-3) to 1.94 mg/L (St-6) at all the sampling stations. The mean organic orthophosphate concentrations ranged from 0.004 (St-1 and St-2) to 0.113 mg/L (St-6) at all the sampling stations. Overall, conductivity was observed to range from 31.33 to 70.00 μmhos. Conductivity was highest at the sampling St-7 (70.00 μmhos) which is close to the urban area. This was probably due to the dissociation of inorganic compounds and the releases of heavy metals ions into the river water from urban wastes and other human activities.

As expected, the parameters such as water velocity, DO, ammonia, and pH showed a reverse pattern at the polluted downstream stations of Semenyih River. In particular, the water velocity of St-1 at the upstream recorded the highest value (0.97 m/s), while St-7, at the downstream, recorded the lowest (0.17 m/s). The different altitudes or the inclination of the surface in the direction of flow (Coker, 1968) and the increased discharges of the Semenyih Dam were two possible reasons to account for the notable variations in water velocity. In the case of dissolved oxygen (DO), it must be noted that the contents of the water bodies were well above 6 mg/L, and these were possibly due to the photosynthetic activity at all the sampling stations. In addition, the higher water velocity at the upstream stations (St-1 and St-2) was associated with higher DO as the turbulence waters mixed air into the water bodies. The sampling St-2 had the highest average of the DO (7.97 mg/L), whilst the sampling St-6 was the lowest (6.40 mg/L). Interestingly, the concentration of ammonia was found in a reverse pattern when compared to the concentrations of nitrate and orthophosphate. The sampling stations at the upstream (St-1 and St-2), before merging with Tekala River, were found to have high ammonia concentrations (3.36 mg/L). This particular finding was possibly due to the bottom-released water of Semenyih Dam which contained high concentration of ammonia. After joining the Tekala River, dilution seemed to take place as the concentrations of ammonia recorded were low, ranging from 0.48 (St-4 and St-6) to 0.55 mg/L (St-7). On the contrary, the pH values could hardly show any differences, as they were found to range from 6.00 to 6.56.

River depth fluctuated along the sampling stations. The irregular pattern was due to depths where the macrobenthic invertebrates were sampled and were not the deepest parts of the sampling sites. The temperature showed a little fluctuation between the sampling stations, as it ranged from 26.0 to 29.5°C. The highest temperature (29.5°C) was recorded at the sampling St-4 as this site had no shades. On the other hand, the lower temperature at St-5 (26.0°C) was due to the site being located under a roadway bridge and had the lowest temperature amongst all the sampling stations. A slight decrease shown for sampling St-2 (29.0°C) to sampling St-3 (26.7°C) was due to the entry of a cooler and pristine water tributary (Tekala River) to Semenyih River.

TABLE 1
The physico-chemical parameters of the water quality for each station at Sg. Semenyih. All the values represent the means of three samples collected in June, 1997 (individuals/m^2 ± standard error; N= 3)

Station	Wd (m)	Dep (m)	Tem (°C)	Vel (m/s)	DO (m/s)	pH	Con (μmhos)	BOD_3 (mg/L)	Amm (mg/L)	Pho (mg/L)	Nrt (mg/L)	Tbt (FTU)	TSS (mg/L)
1.	11.19 ± 2.13	0.15 ± 0.02	28.0 ± 0.00	0.97 ± 0.12	7.93 ± 0.03	6.49 ± 0.03	35.00 ± 0.00	0.60 ± 0.07	3.36 ± 0.00	0.004 ± 0.001	0.67 ± 0.01	2.33 ± 0.67	4.67 ± 0.67
2.	5.58 ± 0.55	0.20 ± 0.07	29.0 ± 0.00	0.48 ± 0.12	7.97 ± 0.09	6.56 ± 0.04	35.00 ± 0.00	1.23 ± 0.09	3.36 ± 0.00	0.004 ± 0.001	0.70 ± 0.03	2.67 ± 0.33	25.33 ± 18.34
3.	6.50 ± 0.50	0.34 ± 0.02	26.7 ± 0.33	0.82 ± 0.05	7.80 ± 0.06	6.00 ± 0.10	35.00 ± 0.00	1.13 ± 0.18	0.62 ± 0.15	0.012 ± 0.003	0.10 ± 0.01	7.33 ± 0.33	14.00 ± 1.15
4.	11.20 ± 0.80	0.15 ± 0.03	29.5 ± 0.29	0.50 ± 0.11	7.70 ± 0.06	6.13 ± 0.03	31.33 ± 0.67	1.97 ± 0.09	0.48 ± 0.12	0.032 ± 0.003	0.23 ± 0.01	16.00 ± 0.00	25.33 ± 3.53
5.	20.33 ± 0.88	0.31 ± 0.03	26.0 ± 0.00	0.18 ± 0.04	7.43 ± 0.09	6.23 ± 0.01	39.00 ± 0.58	2.40 ± 0.15	0.50 ± 0.13	0.042 ± 0.007	0.60 ± 0.03	37.00 ± 9.54	77.33 ± 17.94
6.	15.67 ± 0.67	0.21 ± 0.04	27.8 ± 0.17	0.25 ± 0.08	6.40 ± 0.17	6.02 ± 0.02	39.00 ± 0.00	2.27 ± 0.12	0.48 ± 0.12	0.113 ± 0.004	1.94 ± 0.00	29.67 ± 0.88	34.00 ± 2.00
7.	32.67 ± 0.33	0.24 ± 0.02	27.8 ± 0.17	0.17 ± 0.01	6.60 ± 0.10	6.12 ± 0.01	70.00 ± 0.00	3.37 ± 0.19	0.55 ± 0.16	0.096 ± 0.005	1.44 ± 0.06	122.00 ± 14.47	146.67 ± 5.70

Wd: River width, Dep: Depth, Tem: Temperature, Vel: Water velocity, Con: Conductivity, Amm: Ammonia, Pho: Phosphate, Nrt: Nitrate, Tbt: Turbidity, TSS: Total suspended solids

Density and Distribution of Macrobenthic Invertebrates

The densities and distributions of macrobenthic invertebrates of Semenyih River are shown in *Fig. 2* through *5*. High diversities were observed at the upstream stations (St-1 to St-3), but at downstream stations (St-5 to St-7), the populations were restricted by sand-bottom substratum and anoxic conditions of silt or polluted mud (Thorne & William, 1997), and therefore, only two species were found at St-7. Meanwhile, only Oligochaeta (*L. hoffmeisteri*) could tolerate unfavourable conditions. In addition, a high density of oligochaetes is a good indication of organic pollution (Slepukhina, 1984). In particular, St-3 recorded the highest number of species. This might originate from the drift from Tekala River which is believed to have a very high species richness due to its pristine conditions and remoteness.

Out of all the taxa recorded, Hirudinea and Oligocheata were the organisms found predominately at the downstream stations (St-5 to St-7) with *L. hoffmeisteri* being the most abundant species. Conversely, the other taxa (Crustacea, Ephemeroptera, Odonata, Gastropoda, Trichoptera, Coleoptera and Diptera) were principally recorded at the upstream sampling stations (Stations 1-3), with Baetidae and *F. m. martensi* being the least abundant at St-1. These species, that seemed to be negligible, were still of paramount importance in contributing towards species richness. It is important to note that some taxa could only be found at St-3. These taxa were Leptophlebiidae, *Hydropsyche annulata*, *Polymorphanisus* sp. and Tipulidae. At St-4, however, only a semi-tolerant bivalve species was found at the sandy bottom site, i.e. *Corbicula javanica*. Details of these macrobenthos compositions can be found in Yap *et al.* (2003a).

Pearson's Correlation Coefficient and Multiple Linear Stepwise Regression Analyses

The Pearson's correlation coefficients between each species and each physico-chemical parameter are presented in Tables 2a, 2b, and 2c. The results indicated that the taxa abundance was significantly influenced by a combination of different physico-chemical parameters. Based on the MLSRA (Tables 3a and 3b), river width, temperature, conductivity, BOD_3 and turbidity were found as good physico-chemical parameters which influence *Pentaneura* spp. at the downstream stations. This finding is supported by the correlations (r values ranging from -0.89 to 0.08) between the abundance of macrobenthic invertebrates and the physico-chemical parameters. Nonetheless, temperature and conductivity were not significantly correlated with *Pentaneura* spp. This was due to the analysis of MLSRA which had generalized the most influential ones and excluded the less important parameters regardless of the significant correlated parameters found in the Pearson's correlation coefficient.

The somewhat non-correspondence between the Pearson's correlation coefficient and the MLSRA of their parameters found was attributed to the fact that the procedures of the MLSRA had enumerated all the physico-chemical parameters into determination for the most important variables and this statistical procedures are more logical in practice since the nature consists of complicated abiotic factors. Once again, only the good parameters were shown, whereas the less influential ones were eliminated. As for the Pearson's correlation coefficient, every single parameter was found to have correlated with a macrobenthic invertebrates, one at a time.

Macrostemum similior was found to be negatively correlated with the parameters such as river width, BOD_3, orthophosphate, turbidity, and total suspended solids. On the contrary, *Macrostemum* sp. was positively correlated with water velocity, DO, pH, and ammonia. As high DO was usually found at clean stations, it is therefore plausible to make a statement that *Macrostemum* sp. is a good bioindicator for that particular system. Based on the MLSRA, the most influential parameters for the density of *Macrostemum* sp. were river width, temperature, pH, conductivity, BOD_3, and ammonia.

At St-3, where the highest number of species was found at all the stations, river

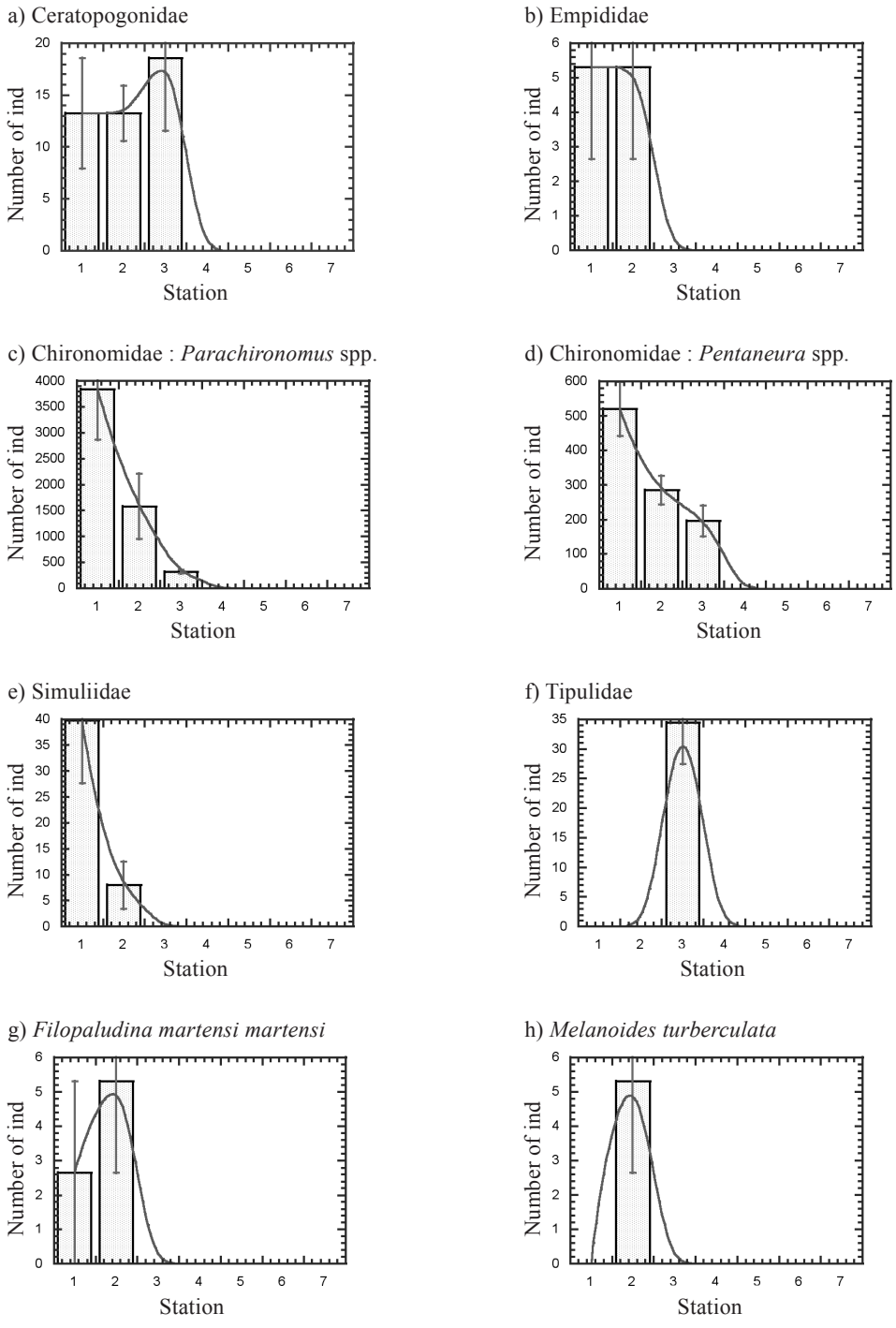

*Fig. 2: The mean number of individual (ind/m² ± SE; N= 3) Diptera (Ceratopogonidae, Simuliidae, Chironomidae, Empididae and Tipulidae) and Gastropoda (*F. m. martensi *and* M. turberculata*) at all the sampling stations at Sg. Semenyih in June, 1997*

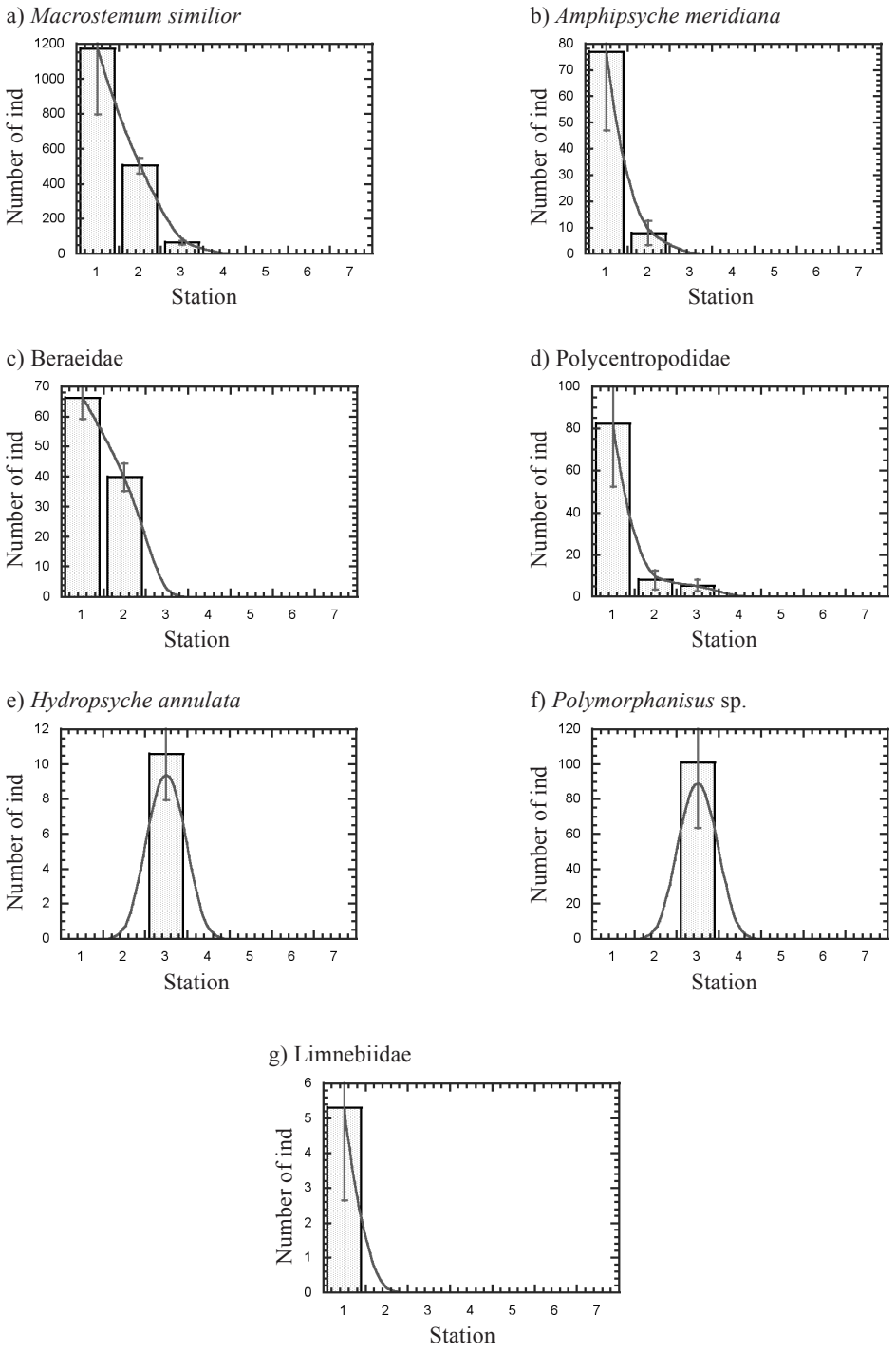

*Fig. 3: The mean number of individual (ind/m² ± SE; N= 3) Trichoptera (*M. similior*,* A. meridiana*, Beraeidae, Polycentropodidae,* H. annulata *and* Polymorphanisus *sp.) and Coleoptera (Limnebiidae) at all the sampling stations at Sg. Semenyih in June, 1997*

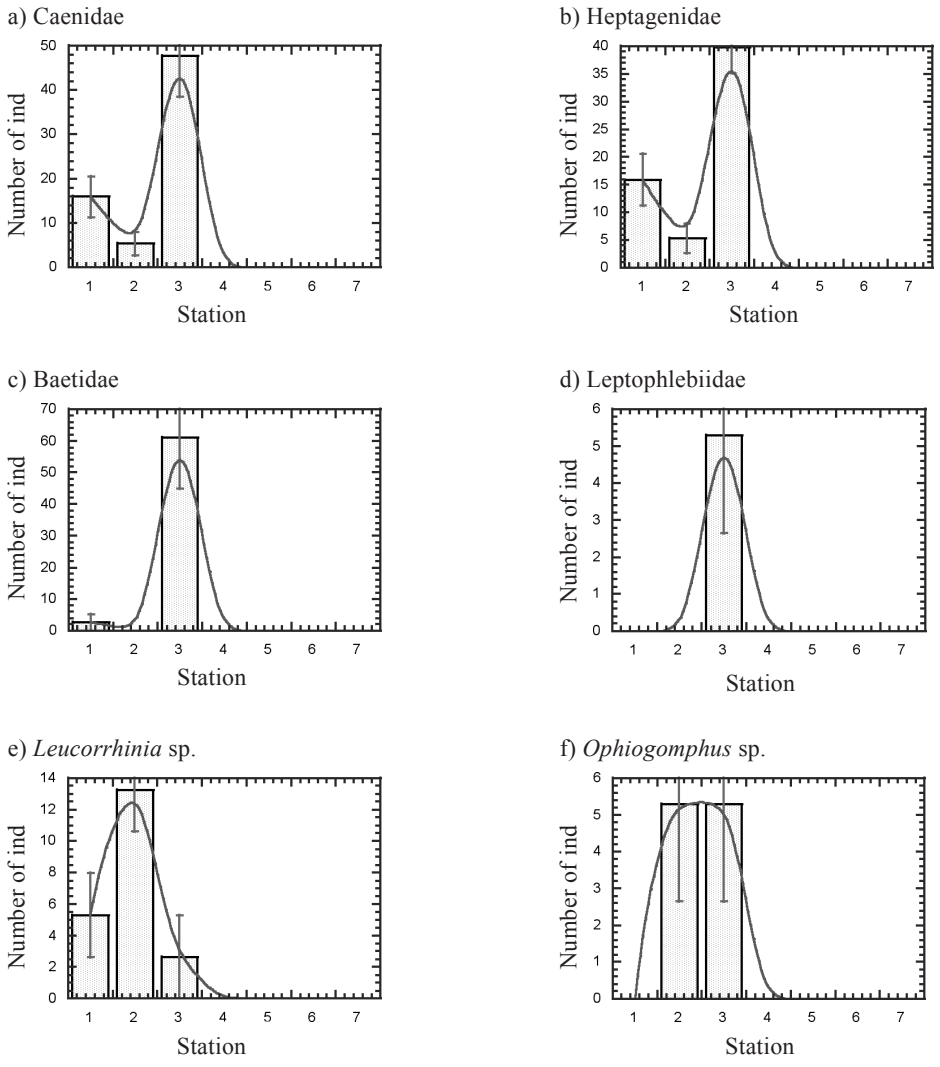

*Fig. 4: The mean number of individual (ind/m² ± SE; N= 3) Ephemeroptera (Caenidae, Heptagenidae, Baetidae and Leptophlebiidae) and Odonata (*Leucorrhinia *sp. and* Ophiogomphus *sp.) at all the sampling stations of Sg. Semenyih in June, 1997*

depth, pH, conductivity, nitrate, and total suspended solids were observed and found to be the most influential parameters by the MLSRA to Baetidae, although only river depth and nitrate were significantly correlated with the density and presence of Baetidae (Table 2a). Meanwhile, *Polymorphanisus* sp. was significantly influenced by river width, temperature, DO, conductivity, nitrate and turbidity, in which the only significantly correlated parameter was nitrate (Table 2b). *Heptageniidae* was significantly influenced by river depth, water velocity, DO, conductivity, BOD_3, nitrate and turbidity, in which the only significantly correlated parameters were water velocity, DO and BOD_3 (Table 2a).

Among the dominant species, *Parachironomus* spp. was positively correlated to water velocity, DO, pH, and ammonia (Table 2b). The species was negatively correlated to

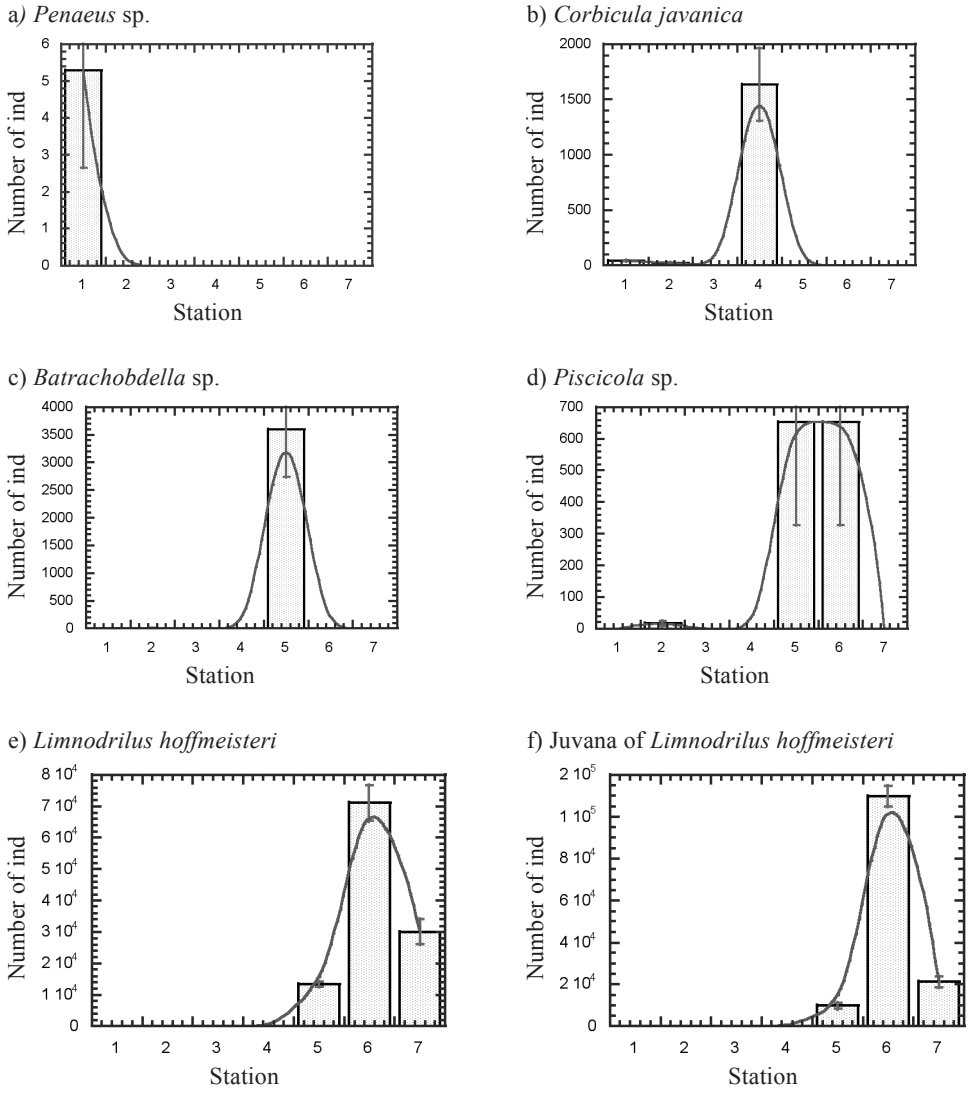

*Fig. 5: The mean number of individual (ind/m² ± SE; N= 3) Crustacea (*Penaeus *sp.), Bivalvia (*C. javanica*), Hirudinea (*Batrachobdella *sp. and* Piscicola *sp.) and Oligochaeta (*L. hoffmeisteri*) at all the sampling stations of Sg. Semenyih in June, 1997*

river width, BOD_3, orthophosphate, turbidity, and total suspended solids. Based on the MLSRA, the non-significant correlations (temperature and conductivity) were identified as the influential parameters, whilst water velocity and total suspended solids were eliminated.

As stated earlier, the only dominant species found at St-4 was *C. javanica*. The species was highly correlated with river depth, temperature, DO and conductivity, as shown in Table 2c. Izzatullayev (1992) studied the aquatic mollusks of Central Asia as water quality indicators. Amongst the frequencies of the occurrence of indicator mollusks in the waters of various types, genera *Corbicula* (*C. cor*, *C. fluminalis* and *C. purpurea*) appeared to be good indicators of oligosaprobic (very slightly polluted water). This raised the possibility for us to make a

TABLE 2a
The Pearson's correlation coefficients analysis between the physico-chemical factors (with log10 transformed) and the distributions of each macrobenthic invertebrate (with fourth-root transformed) at the 7 stations of Sg. Semenyih in June, 1997

	Wd (m)	Dep (m)	Tem (°c)	Vel (m/s)	DO (mg/L)	pH	Con (μmhos)	BOD_3 (mg/L)	Amm (mg/L)	Pho (mg/L)	Nrt (mg/L)	Tbt (FTU)	TSS (mg/L)
Piscicola sp.	0.16^{ns}	0.14^{ns}	-0.28^{ns}	0.60^{**}	-0.18^{ns}	-0.02^{ns}	-0.06^{ns}	0.21^{ns}	-0.23^{ns}	-0.28^{ns}	0.37^{ns}	0.19^{ns}	0.26^{ns}
Batrachobdella sp.	0.26^{ns}	0.39^{ns}	-0.60^{**}	-0.45^{*}	0.09^{ns}	0.13^{ns}	-0.04^{ns}	0.27^{ns}	-0.16^{ns}	0.11^{ns}	0.04^{ns}	0.20^{ns}	0.34^{ns}
Penaeus sp.	0.03^{ns}	0.40^{ns}	0.05^{ns}	0.47^{*}	0.26^{ns}	0.44^{*}	-0.15^{ns}	-0.53^{*}	0.47^{*}	-0.39^{ns}	0.05^{ns}	-0.48^{*}	-0.47^{*}
Caenidae	-0.66^{**}	-0.00^{ns}	-0.16^{ns}	0.76^{***}	0.58^{**}	0.21^{ns}	-0.35^{ns}	-0.77^{***}	0.47^{*}	-0.75^{***}	-0.56^{**}	-0.70^{***}	-0.76^{***}
Baetidae	-0.49^{*}	0.45^{*}	-0.40^{ns}	0.49^{*}	0.33^{ns}	-0.31^{ns}	-0.22^{ns}	-0.41^{ns}	-0.02^{ns}	-0.32^{ns}	-0.70^{***}	-0.26^{ns}	-0.34^{ns}
Heptageniidae	-0.65^{**}	0.16^{ns}	-0.15^{ns}	0.70^{***}	0.57^{**}	0.23^{ns}	-0.35^{ns}	-0.78^{***}	0.48^{*}	-0.72^{***}	-0.53^{*}	-0.69^{***}	-0.64^{**}
Leptoplebiidae	-0.38^{ns}	0.42^{***}	-0.23^{***}	0.37^{***}	0.21^{***}	-0.42^{***}	-0.15^{***}	-0.25^{ns}	-0.18^{ns}	-0.10^{ns}	-0.57^{***}	-0.14^{ns}	-0.20^{ns}
Leucorrhinia sp.	-0.60^{**}	-0.17^{ns}	0.30^{ns}	0.44^{*}	0.55^{**}	0.68^{***}	-0.29^{ns}	-0.55^{**}	0.77^{***}	-0.74^{***}	-0.08^{ns}	-0.72^{***}	-0.50^{*}
Ophiogomphus sp.	-0.55^{*}	0.22^{ns}	-0.04^{ns}	0.25^{ns}	0.36^{ns}	0.27^{ns}	-0.22^{ns}	-0.19^{ns}	0.33^{ns}	-0.43^{*}	-0.41^{ns}	-0.40^{ns}	-0.18^{ns}
Macrostemum similior	-0.68^{***}	-0.22^{ns}	0.17^{ns}	0.71^{***}	0.67^{***}	0.71^{***}	-0.37^{ns}	-0.89^{***}	0.86^{***}	-0.91^{***}	-0.19^{ns}	-0.87^{***}	-0.78^{***}

Note: Wd: River width, Dep: Depth, Tem: Temperature, Vel: Water velocity, Con: Conductivity, Amm: Ammonia, Pho: Phosphate, Nrt: Nitrate, Tbt: Turbidity, TSS: Total suspended solids. Values given are the correlation coefficients (r) and their levels of significance (ns $p > 0.05$, *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

TABLE 2b
The Pearson's correlation coefficients analysis between the physico-chemical factors (with log10 transformed) and the distributions of each macrobenthic invertebrate (with fourth-root transformed) in the 7 stations of Sg. Semenyih in June, 1997

	Wd (m)	Dep (m)	Tem (°c)	Vel (m/s)	DO (mg/L)	pH	Con (μmhos)	BOD_3 (mg/L)	Amm (mg/L)	Pho (mg/L)	Nrt (mg/L)	Tbt (FTU)	TSS (mg/L)
A. meridiana	-0.36^{ns}	-0.52*	0.22^{ns}	0.52*	0.46*	0.73***	-0.25^{ns}	-0.76***	0.78***	-0.72***	0.11^{ns}	-0.69***	-0.77***
H. annulata	-0.44*	0.49*	-0.42^{ns}	0.43^{ns}	0.26^{ns}	-0.42^{ns}	-0.19^{ns}	-0.27^{ns}	-0.16^{ns}	-0.22^{ns}	-0.74***	-0.18^{ns}	-0.22^{ns}
Polymorphanisus sp.	-0.43^{ns}	0.47*	-0.42^{ns}	0.43^{ns}	0.27^{ns}	-0.38^{ns}	-0.19^{ns}	-0.25^{ns}	-0.13^{ns}	-0.22^{ns}	-0.75***	-0.18^{ns}	-0.21^{ns}
Beraeidae	-0.47*	-0.44*	0.35^{ns}	0.50*	0.54*	0.88***	-0.29^{ns}	-0.74***	0.92***	-0.82***	0.13^{ns}	-0.81***	-0.66**
Polycentropodidae	-0.45*	-0.19^{ns}	0.11^{ns}	0.62**	0.52*	0.54*	-0.31^{ns}	-0.83***	0.69***	-0.73***	-0.14^{ns}	-0.77***	-0.70***
Limnebiidae	0.03^{ns}	-0.40^{ns}	0.05^{ns}	0.47*	0.26^{ns}	0.44*	-0.15^{ns}	-0.53*	0.47*	-0.39^{ns}	0.05^{ns}	-0.48*	-0.47*
Tipulidae	-0.44*	0.49*	-0.42^{ns}	0.43^{ns}	0.26^{ns}	-0.43^{ns}	-0.20^{ns}	-0.28^{ns}	-0.16^{ns}	-0.22^{ns}	-0.74***	-0.18^{ns}	-0.22^{ns}
Simuliidae	-0.36^{ns}	0.55**	0.24^{ns}	0.54*	0.47*	0.75***	-0.25^{ns}	-0.75***	0.80***	-0.73***	0.11^{ns}	-0.71***	-0.77***
Pentaneura spp.	-0.73***	-0.13^{ns}	0.08^{ns}	0.76***	0.68***	0.59**	-0.40^{ns}	-0.89***	0.78***	-0.92***	-0.34^{ns}	-0.88***	-0.79***
Parachironomus spp.	-0.68***	-0.24^{ns}	0.14^{ns}	0.74***	0.67***	0.67***	-0.38^{ns}	-0.91***	0.84***	-0.92***	-0.22^{ns}	-0.87***	-0.82***
Ceratopogonidae	-0.78***	0.02^{ns}	0.01^{ns}	0.74***	0.68***	0.46*	-0.39^{ns}	-0.85***	0.69***	-0.89***	-0.43^{ns}	-0.83***	-0.72***

Note: Wd: River width, Dep: Depth, Tem: Temperature, Vel: Water velocity, Con: Conductivity, Amm: Ammonia, Pho: Phosphate, Nrt: Nitrate, Tbt: Turbidity, TSS: Total suspended solids. Values given are the correlation coefficients (r) and their levels of significance (^{ns} $p > 0.05$, $^{*}p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

TABLE 2
The Pearson's correlation coefficients analysis between the physico-chemical factors (with log10 transformed) and the distributions of each macrobenthic invertebrate (with fourth-root transformed) at the 7 stations of Sg. Semenyih in June, 1997

	Wd (m)	Dep (m)	Tem (°c)	Vel (m/s)	DO (mg/L)	pH	Con (μmhos)	BOD_3 (mg/L)	Amm (mg/L)	Pho (mg/L)	Nrt (mg/L)	Tbt (FTU)	TSS (mg/L)
Empididae	-0.28^{ns}	-0.29^{ns}	0.28^{ns}	0.48^{*}	0.44^{*}	0.67^{***}	-0.22^{ns}	-0.55^{*}	0.71^{***}	-0.58^{**}	0.09^{ns}	-0.62^{**}	-0.39^{ns}
F. m. martensi	-0.37^{ns}	-0.50^{*}	0.30^{ns}	0.28^{ns}	0.34^{ns}	0.61^{**}	-0.19^{ns}	-0.33^{ns}	0.60^{**}	-0.60^{**}	0.08^{ns}	-0.59^{**}	-0.49^{*}
M. turberculata	-0.41^{ns}	0.01^{ns}	0.32^{ns}	0.17^{ns}	0.33^{ns}	0.45^{*}	-0.15^{ns}	-0.20^{ns}	0.47^{*}	-0.39^{ns}	0.06^{ns}	-0.35^{ns}	-0.04^{ns}
C. javanica	-0.29^{ns}	-0.63^{**}	0.73^{***}	0.39^{ns}	0.47^{*}	0.23^{ns}	-0.51^{*}	-0.22^{ns}	0.18^{ns}	-0.27^{ns}	-0.33^{ns}	-0.33^{ns}	-0.33^{ns}
L. *hoffmeisteri*	0.77^{***}	0.26^{ns}	-0.35^{ns}	-0.81^{***}	-0.91^{***}	-0.43^{*}	0.63^{**}	0.72^{***}	-0.54^{*}	0.84^{***}	0.73^{***}	0.79^{***}	0.70^{***}
L. *hoffmeisteri* (Juvana)	0.71^{***}	0.24^{ns}	-0.32^{ns}	-0.77^{***}	-0.91^{***}	-0.45^{*}	0.56^{**}	0.69^{***}	-0.52^{*}	0.83^{***}	0.73^{***}	0.74^{***}	0.64^{**}

Note: Wd: River width, Dep: Depth, Tem: Temperature, Vel: Water velocity, Con: Conductivity, Amm: Ammonia, Pho: Phosphate, Nrt: Nitrate, Tbt: Turbidity, TSS: Total suspended solids. Values given are the correlation coefficients (r) and their levels of significance (^{ns} $p > 0.05$, $^{*}p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

TABLE 3a

Multiple Linear Stepwise Regression Analysis between the dependent variables (macrobenthic invertebrates with fourth-root transformed) and the independent variables (physico-chemical parameters with log10 transformed) at the 7 stations of Sg. Semenyih in June, 1997

Taxa	a	b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	r^2	r	F	P
Emp	0.41	1.13 Amm								0.50	0.71	19.08	P< 0.001
Pis	15.19	-9.66 Vel	-0.55 Con	-5.36 BOD_3						0.74	0.86	16.28	P< 0.001
Leu	3.26	-1.60 Wd	1.46 Amm	0.57 TSS						0.70	0.84	13.26	P< 0.001
Oph	4.52	-2.38 Wd	0.57 Amm	0.98 TSS						0.49	0.70	5.51	P< 0.01
Pen	-1.17	2.50 Wd	0.79 Vel	-0.95 Tbt						0.83	0.91	27.17	P< 0.001
MT	3.65	-1.62 Wd	0.88 Amm	1.04 TSS						0.65	0.81	10.37	P< 0.001
Lim	-1.17	2.50 Wd	0.79 Vel	-0.95 Tbt						0.83	0.91	27.17	P< 0.001
Fmm	4.08	-1.22 Dep	-8.96 DO	1.71 BOD_3	-1.67 Pho					0.71	0.84	9.74	P< 0.001
Polyc	-3.92	5.83 Con	-3.08 BOD_3	1.04 Pho	-2.00 Tbt					0.82	0.91	17.75	P< 0.001
Lep	12.60	0.78 Dep	-14.91 pH	-0.82 BOD_3	-0.36 Nrt					0.58	0.76	5.51	P< 0.01
Bat	92.61	-93.02 Tem	-3.51 Vel	88.95 pH	-15.58 Con	2.69 TSS				0.82	0.91	14.06	P< 0.001
HA	38.02	-9.71 Tem	-13.47 DO	-17.67 pH	-1.01 Pho	-1.24 Nrt				0.89	0.94	24.20	P< 0.001
Pent	15.90	-3.15 Wd	-17.30 Tem	11.09 Con	-3.13 BOD_3	-2.40 Tbt				0.97	0.98	104.82	P< 0.001
Bae	7.63	3.01 Dep	-18.04 pH	3.96 Con	-1.27 Nrt	-1.62 TSS				0.86	0.93	19.15	P< 0.001

Note: Pis: *Piscicola* sp., Bat: *Batrachobdella* sp., Pen: *Penaeus* sp., Bae: Baetidae, Lep: Leptophlebiidae, Leu: *Leucorrhinia* sp., HA: *Hydropsyche annulata*, Oph: *Ophiogomphus* sp., Polyc: Polycentropodidae, Lim: Limnebiidae, Pent: *Pentaneura* spp., Emp: Empididae, Fmm: *Filopaludina martensi martensi*, MT: *Melanoides turberculata*,
Wd: River width (m), Dep: Depth (m), Tem: Temperature (°C), Vel: Water velocity (m/s), Con: Conductivity (µhmos), Amm: Ammonia (mg/L), Pho: Phosphate (mg/L), Nrt: Nitrate (mg/L), Tbt: Turbidity (FTU), TSS: Total suspended solids (mg/L)

TABLE 3b

The Multiple Linear Stepwise Regression Analysis between the dependent variables (macrobenthic invertebrates with fourth-root transformed) and independent variables (physico-chemical parameters with log10 transformed) at the 7 stations of Sg. Semenyih in June, 1997

Taxa	a	b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	r^2	r	F	P
Cera	27.02	1.23 Dep	-5.72 DO	-29.84 pH	0.81 Amm	-2.02 Pho				0.93	0.96	39.54	P< 0.001
Tipu	45.39	0.85 Dep	-8.25 Tem	-15.83 DO	-27.07 pH	-1.39 Pho	-1.45 Nrt			0.91	0.95	23.48	P< 0.001
LM	228.87	-106.8 Tem	-3.48 Vel	-63.72 DO	-5.13 Con	3.32 Pho	5.95 Nrt			0.99	0.99	232.39	P< 0.001
LMj	261.75	-105.7 Tem	-2.58 Vel	-87.98 DO	-13.36 Con	3.19 Pho	6.54 Nrt			0.98	0.99	121.66	P< 0.001
MS	-21.19	-3.09 Wd	-7.72 Tem	34.54 pH	7.22 Con	-5.84 BOD_3	1.30 Amm			0.99	0.99	178.26	P< 0.001
Polym	46.84	-1.99 Wd	-22.45 Tem	-22.57 DO	5.03 Con	-2.83 Nrt	-0.82 Tbt			0.94	0.97	38.55	P< 0.001
Ber	-29.21	6.09 Tem	26.46 pH	-1.87 BOD_3	0.53 Amm	-0.50 Pho	0.85 Nrt			0.99	0.99	174.85	P< 0.001
Hep	9.30	1.01 Dep	-1.11 Vel	-16.84 DO	5.19 Con	-3.17 BOD_3	-2.05 Nrt	-1.58 Tbt		0.91	0.95	17.79	P< 0.001
Cae	22.68	-1.10 Wd	-18.41 Tem	-7.81 DO	7.02 Con	-1.37 Nrt	-0.95 Tbt	-1.38 TSS		0.98	0.99	55.25	P< 0.001
AM	-39.08	0.57 Wd	41.26 pH	1.55 Con	-2.45 BOD_3	0.33 Nrt	1.20 Tbt	-1.93 TSS		0.98	0.99	98.28	P< 0.001
Simu	-34.00	0.73 Wd	-0.68 Dep	34.39 pH	2.10 Con	-1.83 BOD_3	0.65 Tbt	-1.45 TSS		0.97	0.98	65.70	P< 0.001
Cj	-136.9	4.46 Wd	-2.46 Dep	86.62 Tem	25.37 DO	-10.52 Con	-1.41 Nrt	1.06 Tbt		0.95	0.97	38.13	P< 0.001
Para	-24.50	-4.02 Wd	-19.08 Tem	1.04 Vel	50.54 pH	11.85 Con	-6.22 BOD_3	1.09 Amm	-1.18 TSS	0.99	0.99	212.10	P< 0.001

Note: Tipu: Tipulidae, Simu: Simuliidae, Para: *Parachironomus* spp., Cera: Ceratopogonidae, Cj: *Corbicula javanica*, LM: *Limnodrilus hoffmeisteri*, LMj: *L. hoffmeisteri* (Juvana), Cae: Caenidae, Hep: Heptageniidae, MS: *Macrostemum similior*, AM: *Amphipsyche meridiana*, Polym: *Polymorphanisus* sp., Ber: Beraeidae,
Wd: River width, Dep: Depth, Tem: Temperature, Vel: Water velocity, Con: Conductivity, Amm: Ammonia, Pho: Phosphate, Nrt: Nitrate, Tbt: Turbidity, TSS: Total suspended solids

conclusion that St-4 was a slightly polluted site as *C. javanica* could still survive in this system. However, since our knowledge of freshwater mollusks in our local ecoregion is extremely scarce, further studies are still needed to confirm this hypothesis. Based on the MLSRA, river width, nitrate and turbidity were also included in this study, apart from the above-mentioned parameters.

At the downstream stations (Stations 5-7), Oligochaeta were predominately found. In general, all the physico-chemical parameters, including low DO (Lang, 1985), seemed to be significantly correlated to *L. hoffmeisteri*, except for river depth and temperature. As for *L. hoffmeisteri* (Table 2c), the density and distribution were found to significantly and positively correlate with river width, conductivity, BOD3, concentrations of phosphate and nitrates, turbidity and TSS of the river waters. On the other hand, their densities were shown to be significantly ($p<0.001$) but negatively correlated with water velocity and DO. All the above correlation pairings indicated that *L. hoffmeisteri* is a good bioindicator of the polluted condition at Semenyih River.

It must be noted that the factors identified by the MLSRA are in fact the most important factors amongst all the physico-chemical parameters recorded in this study, and therefore, it was assumed that there are cause-and-effect relationships between them. The researcher also judge that other factors might presumably be contributive to the dependent variable (macrobenthic invertebrates) and those factors were unfortunately not included in this study.

CONCLUSION

Based on the correlation analysis and MLSRA, river width, total suspended solid, turbidity, BOD_3, orthophosphate, nitrate and conductivity were found to have increased with the increasing distance from Semenyih Dam (St-1). On the contrary, water velocity, DO, ammonia and pH showed a reverse pattern. In this study, BOD_3, orthophosphate, total suspended solids, and turbidity were identified as the most important correlates with the community diversity for macrobenthic invertebrates amongst the 13 physico-chemical parameters using the MLSRA. Therefore, the positive relationships between the distribution of sensitive bioindicator species, as well as good water quality and the negative relationships between the distribution of resistant bioindicator species and poor water quality suggest the value of using bioindicator species for Malaysian rivers.

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Carcass Compositions in Three Different Breeds of Chicken and Their Correlation with Growth Performance

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ABSTRACT

This study was undertaken with the aim to determine the carcass composition of three breeds of chicken and their correlation with growth performance. For this purpose, fifty Red Jungle Fowl (*Gallus gallus Spadiceus*), fifty Malaysian indigenous chickens (*Gallus gallus Domesticus*) and fifty broiler chickens (ROSS) were used in this study. The chickens in each group were sacrificed at 1, 10, 20, 56, and 120 days post-hatching. The results showed that there were significant differences in the parameters measured between the high performance breed (commercial broilers), and the lower performance breeds (Red Jungle Fowl and Malaysian Indigenous chickens), although they were reared under the same environment and received the same feed, management and other facilities. Meanwhile, relative whole carcass weight, bone and fat weights in the commercial broiler were the highest compared to indigenous chickens and red jungle fowl at ($p<0.05$).

Keywords: Red Jungle Fowl, Malaysian indigenous chicken, commercial broiler, carcass composition

INTRODUCTION

The red jungle fowl is known to be the ancestor of all domestic fowl. It is classified as omnivorous, slow growth rate and the range of the species stretches from northeast India eastwards across Southern China and down into Malaysia and Indonesia (Condon, 2006). The Malaysian indigenous chicken or known as village chicken arrived from crossbreeding between red jungle fowl and mixed exotic domestics breeds that has been brought by the Europeans, mainly British (Petersen *et al.*, 1991). Current commercial broiler chicken strains are a result of successful selection programs for rapid growth and body conformation, especially favouring the breast muscles which could have significant economic impact (Scheurmann *et al*., 2003).

In general, chicken growth is well described as a sigmoid curve with an initial exponential development phase, an intermediate or transitory phase, and a final phase of inhibited growth that consists of a gradual reduction in the growth rate, following an asymptotic increase in the body weight (Aguilar *et al*., 1983). Meanwhile, the differences in the selection criteria among the primary breeders, favouring specific environments or genotypes, may affect the chronology of events during the growth process (Scheurmann *et al*., 2003).

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Up to this date, there have been very few data on the carcass composition of the red jungle fowl and the Malaysian indigenous chickens documented elsewhere. Thus, this study was undertaken with the aim to define the carcass composition of the Red Jungle Fowl (*Gallus gallus Spadiceus*) and Malaysian Indigenous Chicken (*Gallus gallus Domesticus*). The results were compared with the commercial broiler chicken.

MATERIALS AND METHODS

Animals

Three breeds of chicken, known to differ greatly in the growth rates, were used in this study. These breeds were commercial broiler breed (ROSS), Malaysian indigenous chicken (*Gallus gallus Domesticus*) and Red jungle fowl (*Gallus gallus Spadiceous*). A total of 150 chickens, consisting of 50 chickens in each breed, were used in this study. The three breeds of chicken were reared separately in three different cages in an experimental house from their DOC till end of the experiment. The chickens were given commercial feed and drinking water *ad libitum*. For the Malaysian indigenous chickens, the eggs were obtained from the same sources in Jenderam Hulu, Sepang, Selangor, and were hatched in the laboratory using a hatchery. This is similar for the Red jungle fowl, whereby the eggs were obtained from the Centre of Animal Conservation in Jenderam Hulu, Sepang, Selangor, and were hatched in the laboratory using a hatchery. The commercial broiler chickens were obtained from CP (M) Private Limited hatchery in Taiping, Perak. All the chickens for each breed were sacrificed by intravenous (cutaneous ulnar vein) administration of sodium pentobarbitone (80mg/ kg) (Michell & Smith, 1991) at days 1, 10, 20, 56, and 120 post-hatching.

Carcass Composition Measurement

The live weight of each chicken was taken and recorded prior to euthanasation. Upon euthanasation, the skin, viscera, head and legs were removed leaving the whole carcass. The legs were cut at the end of tibiotarsal bone, while the head and neck were removed at the point of 3rd cervical vertebra, and the wings were cut at the point of humerocarpal band. The whole carcass for each chicken was weighted and recorded. The breast circumference of each chicken was taken by using a nylon string and then matching it to the ruler scale, and recorded. This was followed by removing the fat from the carcass, weighted and recorded. After removing the fat, the meat and bone were separated, weighted and recorded.

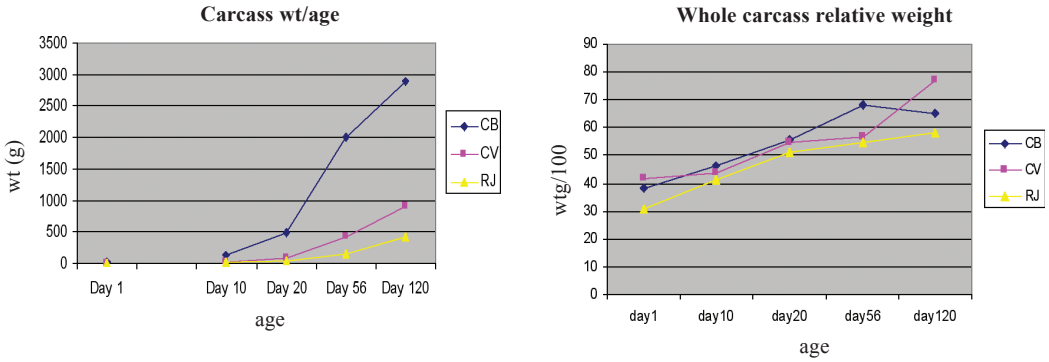
Statistical Analysis

In this study, the collected data were analyzed statistically using SPSS 17.0 software. The results were illustrated using tables, bar graphs and line chart.

RESULTS

The result of the mean whole carcass weight for the three breeds of chickens showed that CB has the highest whole carcass weight at all evaluated ages, followed by CV and RJ (*Fig. 1A*). Although the mean weights of the whole carcass at different ages were different, the result showed that there was no significant difference ($p<0.05$) at the early age (between day 1 and day 10 post-hatching) for all the three breeds. In general, the mean relative weight for all the three breeds showed almost similar increment pattern from day 1 to day 120 post-hatch (*Fig. 1B*). It is important to note that the relative whole carcass weight in all the three breeds increased with age, while the increments were significantly different ($P<0.05$) between the age intervals.

The mean meat weight and bone weight showed almost similar patterns as the whole carcass weight, whereby CB had the highest content of meat and bones at all the ages evaluated, followed by CV and RJ (*Figs. 2A* and *3A*). Although the meat content increased according to age, the differences were not significant at day 1 and day 10 post-hatching. However, a significant difference ($p < 0.05$) was

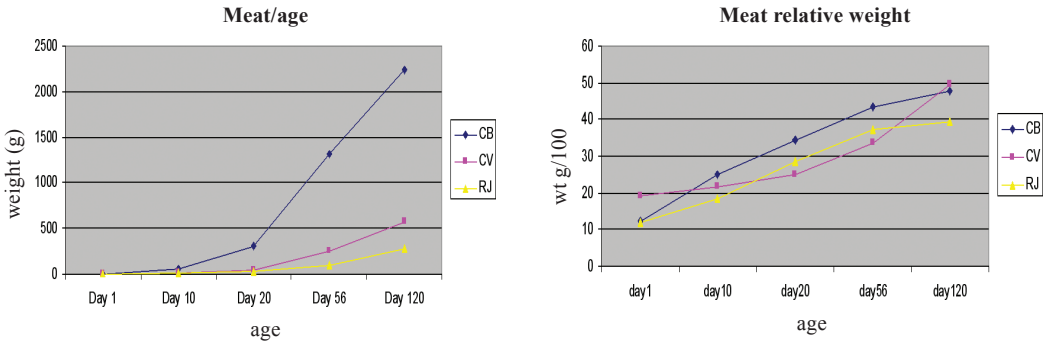


(A) (B)

Fig. 1: The mean whole carcass weight (A), and the mean relative whole carcass weight (B) vs. age at post-hatching for the three breeds of chicken

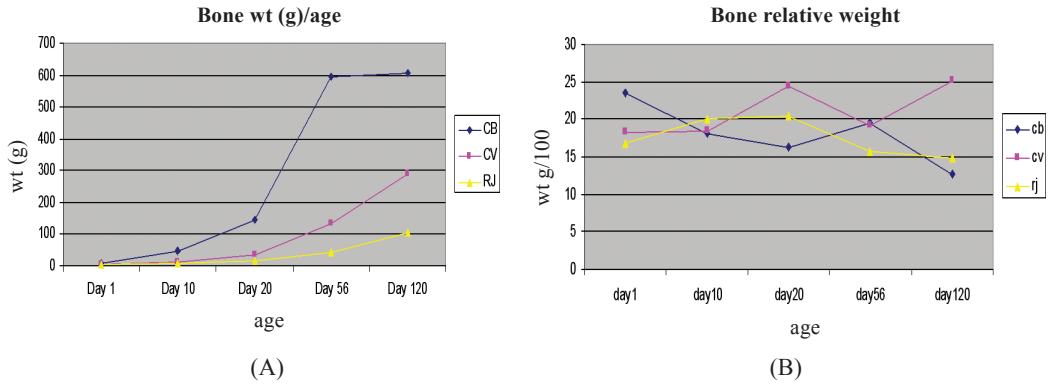
shown at the later ages. As for the mean relative weight, all the three breeds generally showed almost a similar increment pattern of mean relative meat weight from day 1 to day 120 post-hatch (*Fig. 2B*). Meanwhile, the CB showed the highest mean meat relative weight, except at days 1 and 120 of post-hatch, whereby the CV was the highest at these points. The mean bone relative weight revealed a significant difference between the breeds at all ages, except at day 10 post-hatch. In general, the bone relative weight for the CB showed a decreasing pattern from day 1 to day 120, with the CV showing an increasing pattern, and RJ showing almost a constant pattern (*Fig. 3B*).

The result for the mean fat weight showed that the CB had the highest content of fat at all different ages evaluated, followed by CV and RJ (*Fig. 4A*). Within the breeds, although the fat content was increased when the age increased, the differences were not significant between day 1 and day 10 post-hatching for CB and CV. Nonetheless, the significant difference ($p<0.05$) was observed at the later ages. The content of fat in the RJ, on the contrary, remained insignificantly different until day 56 of post-hatching. In general, the RJ showed the lowest fat relative weight among the three breeds (*Fig. 4B*), while the CV had the highest fat relative weight at days 10 to 56, but lowest at



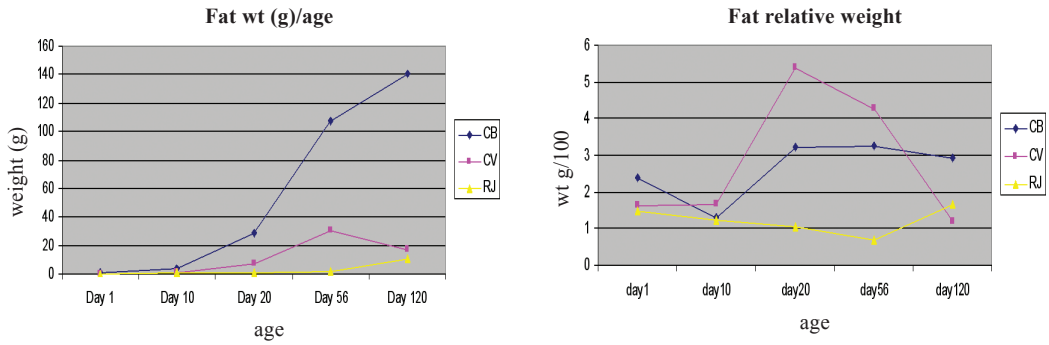
(A) (B)

Fig. 2: The mean weight of meat (A) and the mean relative weight of meat (B) vs. age post-hatching for the three breeds of chicken



(A) (B)

Fig. 3: The mean weight of the bones (A) and the mean relative weight of the bones (B) vs. age at post-hatching for the three breeds of chicken

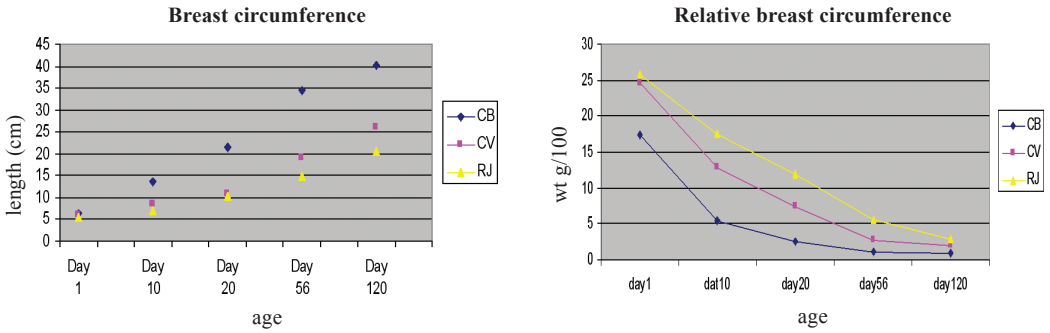


(A) (B)

Fig. 4: The mean weight of fat (A) and the mean relative fat weight (B) vs. age post-hatching for the three breeds of chicken

day 120 post-hatch. The CB had the highest mean fat relative weight at day 1 and day 120 of post-hatching. Both the CV and RJ showed no significant different at days 1, 10 and 120, while CV showed higher mean fat relative weight than the RJ, except at day 120 of post-hatching.

The results for the mean breast circumference showed that the CB was the highest at all different ages evaluated, and this was followed by the CV and RJ. The differences between the three breeds were significantly different ($p<0.05$), except at days 1 and 20 post-hatching for the CV and RJ (*Fig. 5A*). Even though the breast circumference was higher in the CV as compared to RJ at days 1 and 20 post hatching, the differences between them were not significant ($p>0.05$). Within the breeds, the breast circumference was found to increase with age, while the increments between the age evaluated were significantly different ($p<0.05$). As for the mean breast circumference relative weight, all the three breeds generally showed almost a similar decrement pattern from days 1 till 120 of post-hatching, with the RJ showing the highest, followed by CV and CB (*Fig. 5B*). There were significant differences ($P<0.05$) between the three breeds at all ages evaluated, except at day 1, whereby the CV and RJ showed no significant difference even though the RJ was found to have a higher mean breast circumference relative weight.



(A) (B)

Fig. 5: The mean breast circumference (A), the mean relative breast circumference (B) vs. age at post-hatching for the three breeds of chicken

DISCUSSION

The findings of the present study showed that broiler had significantly higher means for the whole carcass weight, meat weight, bone weight, and fat weight, and this was followed by village chicken and red jungle fowl, although they were reared under the same environment and received the same feed, management and facilities. Meanwhile, skeletal muscle growth and muscle fibre size in animals selected for large body size or rapid growth rate have been reported for several species (Chen *et al*., 2004). The Red Jungle Fowl and Malaysian Indigenous chicken have been known to have had slow growth performances compared to the commercial broiler chickens. The development of the organs, which include skeletal muscle system, seems to be slower and leads to smaller body size. This is why they can produce good quality meat at considerable cost because of the low growth rate and perhaps of low food efficiency. Broilers have been selected and genetically programmed for rapid growth rate, larger body weight and meat production (William & Goldspink, 1978).

In general, chicken growth is well described as a sigmoid curve with an initial exponential development phase, an intermediate or transitory phase, and a final phase of inhibited growth that consists of a gradual reduction in the growth rate following an asymptotic increase in the body weight (Aguilar *et al*., 1983). Therefore, at the early age (day 1 and day 10), there were no significant differences in terms of the whole carcass weight, meat weight, bone weight, and fat weight for all three breeds.

The results showed that the relative whole carcass weight in all the three breeds increased when the age increased with a significant difference at $P<0.05$. In particular, the CB had highest relative whole carcass weight, followed by CV and RJ at days 10, 20 and 56 post-hatch. The findings also showed increased relative weight as in the initial exponential development phase and intermediate or transitory phase but it decreased at later ages as it reached the final growing phase.

CV and RJ also showed a slower meat relative weight compared to the CB which was found to grow slower although there was an increase in the relative meat weight. Thus, further study is still needed to determine the best weight for marketing the CV and RJ. The results in this study revealed that the CB had significantly ($P<0.05$) higher bone content compared to the CV and RJ. The relative bone weight for the CB showed a gradual decrease as the body weight increased, which is best condition for the meat-type as it is designed for fast growing birds. Meanwhile, the CB had the highest content of fat at all ages evaluated, followed by the CV and RJ which had very little fat contents, respectively.

Genetically, the CB has gone through successful selections for meat-type breed as well as utilization of feed and converted it into muscle mass and deposition of fat as compared to the CV and RJ, even though all the three breeds had received similar quantity and quality of feed. The fat content in RJ remained the lowest, except at day 120 of post-hatch. This might be due to their nature, i.e. more active, alert and some general behaviour like exploring and anti-predator. Thus, this finding seems to suggest that the utilization of fat for the energy is higher in the RJ.

As for the carcass composition evaluation, the results generally showed that the CB had the best quality in terms of its relative whole carcass and meat content. However, it was also found to be higher in bone and fat contents. Thus, the CB had higher meat yield as compared to CV and RJ. On the contrary, the RJ had the lowest relative fat content, lowest relative whole carcass weight, lowest meat content, but a higher relative bone weight. Therefore, the CV is always in between the CB and RJ.

CONCLUSION

There were significant differences in the parameters measured between the high performance breed (commercial broilers), and lower performance breeds (namely Red Jungle Fowl and Malaysian Indigenous chickens), although they were reared in the same environment and received the same feed, management and other facilities. Relative to their body weight, commercial broilers had the highest quality in terms of the whole carcass and meat content, but they also had higher bone and fat contents compared to indigenous chickens and red jungle fowl. Thus, the commercial broiler had a higher meat yield compared to the other two breeds; however, this may not necessarily be high quality meat. Red jungle fowl was found to have the lowest relative fat content, lowest relative whole carcass weight, lowest meat content, but higher relative bone weight. Indigenous chickens are always in between the commercial broiler and Red jungle fowl. Thus, this finding revealed that the red jungle fowl possesses more healthy meat as it is low in carcass fat content compared to the other two breeds. Conversely, less meat yield due to small size.

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Antioxidant Study of Garlic and Red Onion: A Comparative Study

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ABSTRACT

Garlic (*Allium sativum* L.) and red onion (*Allium cepa* L.) are among the most common ingredients in Malaysian cuisines. These two *Allium* species are believed to possess medicinal properties including antioxidants. Accordingly, the aim of this study was to compare antioxidant level and activities (i.e. at primary and secondary levels) in both the *Allium* species collected from markets around Kuantan, Pahang Darul Makmur, Malaysia. Current results of total phenolic content (TPC) assay indicate that TPC is higher in red onion (i.e. 53.43 ± 1.72 mg GAE/100g) as compared to garlic (i.e. 37.60 ± 2.31 mg GAE/100g). In addition, EC_{50} value of garlic is lower than that of the red onion, showing a higher free radical scavenging activity in garlic than in red onion. However, the primary antioxidant activities of both the samples are lower than the standard antioxidant, BHA. Therefore, there is a poor relationship between the TPCs and the primary antioxidant activities, indicating that the primary antioxidant activities of both the *Allium* species are not solely due to the phenolic compounds. For secondary antioxidant activity, FIC assay shows that at the highest sample concentration of 1.0 mg/mL**,** red onion has higher ferrous ion chelating effect (i.e. 45.00 ± 1.73%) as compared to garlic (i.e. 43.29 ± 3.89%). Furthermore, both the *Allium* samples show slightly higher ion chelating effect than BHA (i.e. 43.14 ± 1.07%) but lower than EDTA (i.e. 97.9 ± 0.07%). Overall, the findings of the present study show a negative relationship between the results of TPC assay, DPPH radical scavenging activity assay, and FIC assay. To strengthen the validity of the present results and to further assess the potential of both the *Allium* species as natural antioxidant sources, more different assays need to be considered for future work.

Keywords: *Allium cepa* L., *Allium sativum* L., total phenolic content, antioxidant activity

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INTRODUCTION

Modern consumers are becoming more health conscious and more aware of food nutritional value. Among the nutrients, antioxidants are popular due to their ability to prevent many physiological diseases or illnesses. Antioxidant is defined as any substance that when present at low concentration compared to those of an oxidisable substrate significantly delays or prevents oxidation of that substrate (Li *et al*., 2007). Antioxidants are believed to play a very important role in the body defence system against reactive oxygen species (ROS) or free radicals, which are harmful by-products generated during aerobic activity of normal cells. Increasing the intake of dietary antioxidant is believed to assist in maintaining an adequate antioxidant status and therefore, the normal physiological function of living system. According to Tepe *et al*. (2005), antioxidants have great importance in terms of preventing oxidative stress that may cause several generative diseases. Many fruits and vegetables are potentials for decreasing risk effect of several chronic diseases, such as cancer, coronary heart disease and many more.

The *Allium* family has over 700 members; each with different tastes, forms and colours; nonetheless, they are close in biochemical, phytochemical, and neutraceutical contents (Tepe *et al*., 2005). Red onion (*Allium cepa* L.) and garlic (*Allium sativum* L.) are among the important parts of diet in many world populations, and there is also a long-held belief in their health enhancing properties. Among the oldest cultivated plants, garlic and red onion are used as food and for medicinal application as they have been proven to convey many benefits to human due to their long storage and portability. One of the advantages of these *Allium* species is that they could be dried and preserved for several months. Garlic, for instance, has been applied as culinary spice and medicinal herb, and it is an important constituent of the traditional Chinese medicine. On the other hand, onions (including red onions) are native to Eurasia but now grow all over the world. The bulb of onion is used medicinally and onion has been consumed as food for many centuries. In Malaysia, these two *Allium* species are widely used and they are becoming very important components in the preparation of almost all Malaysian cuisines and delicacies.

According to Benkeblia (2005), *Allium* species are revered to possess anti-bacterial and anti-fungal activities, and they contain the powerful antioxidants, sulphur and other numerous phenolic compounds which have aroused great interests for food industries. During the last 20 years, *Allium* spices have been among the most studied vegetables and aroused great interest. In previous studies, garlic is found to exhibit antioxidants activity (Tepe *et al*., 2005) and this fact is set as the foundation for possibilities on the presence of antioxidant activities in other *Allium* species. Apart from that, according to Li *et al*. (2006), synthetic antioxidants such as BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene) need to be replaced with natural antioxidants as several studies showed that a number of synthetic antioxidants were toxic and carcinogenic in animals. Consumers are quite sceptical on the production of any synthetic antioxidant products, and there has always been a good public acceptance when it comes to natural antioxidants. For that reason, the aim of this project was to compare the antioxidant level and activities (i.e. at primary and secondary levels) in garlic and red onion that are available in Malaysian markets in Kuantan, Pahang Darul Makmur.

MATERIALS AND METHODS

Sample Collection

Samples of garlic (*A. sativum* L.) and red onion (*A. cepa* L.) were purchased from the local markets and supermarkets in Kuantan, Pahang Darul Makmur, Malaysia. The samples were randomly selected off the shelves based on their freshness.

Sample Preparation and Extraction

The samples were cut into smaller pieces to ease the drying process. Following the suggestion by Khamsah *et al*. (2006), the drying process was done in a warm room at 45°C (not exceeding 50°C) until all the moisture was gone. However, the findings of our previous antioxidant studies suggest no significant detrimental effects on the total phenolic compounds when drying the samples at 60°C and 70°C (Norshazila *et al*., 2010; Nurliyana *et al*., 2010). Extraction was done using the Soxhlet method (Siddhuraju *et al*., 2002). The samples were weighed at 100 g and inserted into an extraction tube of Soxhlet apparatus. The extracting solvent, i.e. 70% ethanol, was then added into the round flask. The Soxhlet apparatus was then assembled, the heat was set at 60°C and left running for 12 hours. After the extraction process, the samples in the round flask were subjected to rotary evaporation to remove the extracting solvent from the extracts. Finally, the extracts were subjected to freeze drying to remove water from the extracts. The extracts were kept in the dark at 4°C until further uses.

Total Phenolic Content (TPC) Assay

TPC was determined by using Folin Ciocalteu's reagent (Lim *et al*., 2006). 0.3 mL of the extract was introduced into the test tubes, followed by 1.5 mL of Folin Ciocalteu's reagent (diluted 10 times with water) and 1.2 mL of sodium carbonate (7.5% w/v). The tubes were vortexed, covered with parafilm and allowed to stand for 30 min in the dark. The absorption of the samples was taken at 765 nm using Perkin Elmer Lambda 25 UV/Vis spectrophotometer. The TPCs were expressed in gallic acid equivalents (GAE). The gallic acid calibration line has the equation of $y = 9.2402x + 0.0149$ ($R^2 = 0.9971$), where y is the absorbance at 765 nm and x is the concentration of phenolic compounds in mg/g of the sample (the graph is not shown).

1, 1-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity Assay

The free radical scavenging activity of each sample was measured using Perkin Elmer Lambda 25 UV/Vis spectrophotometer, based on the decrease absorbance of ethanolic DPPH solution at 517 nm (Lim *et al*., 2006). Different dilution extracts (0.2 – 1.0 mg/mL), amounting to 1.0 mL, were added to 2.0 mL of the DPPH solution. The samples were then vortexed to thoroughly mix it. The samples were then left to stand in the dark for 30 min. The absorbance readings of the samples were taken at 528 nm using Perkin Elmer Lambda 25 UV/Vis spectrophotometer. Synthetic antioxidant, butylated hydroxyanisole (BHA), was used as a positive control for this assay. The antioxidant activity was expressed as:

$$\% \text{ disappearance} = [(A_{control} - A_{sample})/ A_{control}] \times 100$$

$A_{control}$ = Absorbance reading of the control
A_{sample} = Absorbance reading of the sample

$EC_{50,}$ effective concentration of the extract required for 50% scavenging of DPPH· radicals were calculated from the plotted graph of scavenging activity against sample concentration.

Ferrous Ion Chelating (FIC) Assay

Chelating effects of the samples were measured using the FIC assay. Serial dilutions of the samples were prepared (0.02 mg/mL - 0.1 mg/mL). Next, 50 µL of Ferum chloride ($FeCl_2$, 2 mM) and 1.65 mL of 70% ethanol were added to 500 µL of the sample. The samples were vortexed to mix it thoroughly and were left to stand for 5 min in the dark. After that, 100 µL of ferrozine (5 mM, dissolved in 70% ethanol) was added, and subjected to vortex to mix the samples thoroughly. The samples were once again left to stand in the dark for another 5 min. Finally, the absorbance readings of the samples were measured at 562 nm using Perkin Elmer

Lambda 25 UV/Vis spectrophotometer. Both ethylenediaminetetraacetic acid (EDTA) and BHA were used as the controls. The ability of each sample to chelate ferrous ion was calculated relative to the control consisting of only iron ferrozine, using the following formula:

$$\text{Chelating effect \%} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

$A_{control}$ = Absorbance reading of the control
A_{sample} = Absorbance reading of the sample

Statistical Analysis

All the samples and readings were prepared and measured in triplicate. The results were presented in mean ± standard deviation. As for the data and graphs, they were subjected to analyses using Microsoft® Office Excel 2003.

RESULTS AND DISCUSSION

TPC Assay

Phenolic compounds are the major group contributing to the antioxidant activity of vegetables, fruit, cereals and other plant-based materials. The antioxidant activity of the compounds is partly due to one electron reduction potential that is the ability to act as hydrogen or electron donors (Chan *et al*., 2007). Atoui *et al*. (2005) mention that the antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. Determination of these compounds is usually performed by reacting phenolic compounds with Folin-Ciocalteu's reagent. The Folin-Ciocalteu reagent, Folin's phenol reagent or Folin-Denis reagent is a mixture of phosphomolybdate and phosphotungstate used for the colorimetric assay of phenolic antioxidants and polyphenol antioxidants. Upon this reaction, the two classes of compounds will form a complex known as the phosphomolybdic-phosphotungstic-phenol complex which triggers the formation of a blue colour solution. According to Ajila *et al*. (2007), the more intense the formation of blue colour indicates a higher phenolic content inside the samples. The present study shows that red onion possessed higher TPC (i.e. 53.43 ±1.72 mg GAE/100 g) compared to garlic (i.e. 37.60 ±2.31 mg GAE/100 g), whereby red onion exerted an intense blue solution than the sample solution of garlic (*Fig. 1*). However, Benkeblia (2005) found out that the methanolic extract of garlic (*A. sativum* L. var. Cristo) shows higher TPC than the methanolic extract of red onion (*A. cepa* var. Rouge Amposta), and the difference could be due to the different types of species variants and extracting solvents used in both the studies.

DPPH Assay

DPPH assay is a primary antioxidant activity test that determines the free radical scavenging activity of the respective samples. Primary antioxidant involves the mechanism, whereby

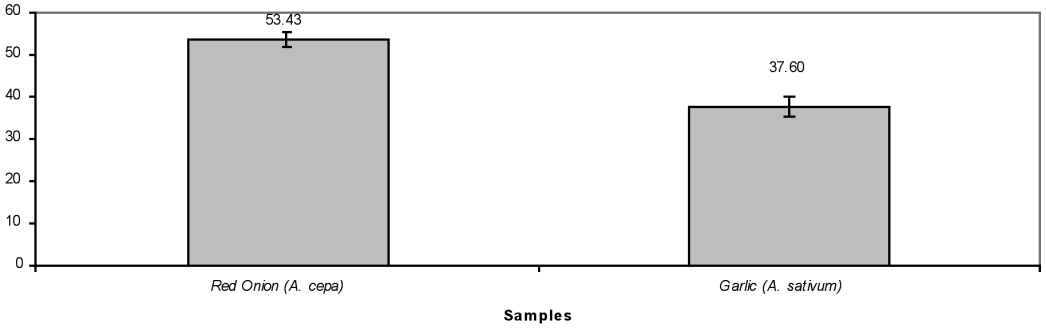

Fig. 1: Level of the total phenolic content in each sample. The results were expressed as gallic acid equivalents (GAE)

it inhibits the oxidation reaction by combining it with the free radicals or reacting hydrogen peroxides. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, it gives rise to the reduced form of the DPPH compound, leading to the reduction of the violet colour.

In the present study, the mechanism of the radical scavenging activity was observed based on the reducing purple colour of DPPH solution. *Fig. 2* shows that free radical scavenging activities of both garlic and red onion were lower than the positive control, BHA; indicating their weak free radical scavenging activities. In terms of IC_{50}, the lowest value is shown by the positive control, BHA (0.16 ±0.01 mg/mL), followed by garlic (0.95 ±0.01 mg/mL) and red onion. However, IC_{50} for red onion could not be directly determined from the graph due to the low percentage of the radical scavenging activities over the measured extract concentrations. Nonetheless, *Fig. 2* clearly suggests that their IC_{50} could be more than 1.0 mg/mL. The results clearly show that garlic has more capability to scavenge the free radicals as compared to red onion, although the primary antioxidant activities of both samples are lower than the standard antioxidant, BHA. Likewise, the findings from Benkeblia (2005), garlic shows higher free radical scavenging activity than red onion over the increasing sample concentrations.

Similar research conducted in other plants and fruit have shown that high radical scavenging activities are usually associated with high TPC. For instance, Lim *et al*. (2006) described that high radical scavenging activity was contributed by the presence of high phenolic content in guava extracts. There are several other studies that share similar results on the contribution of phenolic compounds to the high radical scavenging activity. However, the present study does not support the findings. Therefore, it is suggested that apart from phenolic compounds, there could be other organic compounds contributing to the high radical scavenging effect in garlic, even though its TPC is lower than red onion. Khamsah *et al*. (2006) suggested that free radical scavenging activity is not due to the phenolics only because they found that the antioxidant activity of methanol extract of *Orthosiphon stamineus* was not solely caused by phenolic compounds. IC_{50} data further support that garlic has higher radical scavenging activity (i.e. IC_{50} of garlic= 0.95 mg/mL) than red onion, but the IC_{50} of red onion could not be determined directly from *Fig. 2* due to the low activity over the increasing concentrations. On the contrary, *Fig. 2* clearly suggests that its IC_{50} could be more than 1.0 mg/mL. IC_{50} of both the *Allium* samples are higher than BHA (i.e. IC_{50} of BHA= 0.16 mg/mL), showing their low and weak free radical scavenging activities.

FIC Assay

FIC assay is a common test used to determine the secondary antioxidant activity by observing the reducing purple colour of the reaction solution. The assay mechanism is based on the decrease in the absorbance of iron (II)-ferrozine complex. Meanwhile, secondary antioxidants are also known as the peroxide decomposers, where it inhibits polypropylene oxidation by decomposing hydroperoxide. Secondary antioxidants are responsible for suppressing the formation of radicals and protecting against oxidative damage (Lim *et al*., 2006). Iron-ferrozine complex has the maximum absorbance at 562 nm and large decrease in absorbance indicates strong chelating power. By forming a stable iron (II) chelate, an extract with a high chelating power reduces free ferrous ion concentration, which leads to decrease the extent of Fenton reaction that are implicated in many diseases (Lim *et al*., 2006). The assay determines the effectiveness of the chemical compound in the sample extract in competing ferrous ion with ferrozine.

Iron is known to generate free radicals through the Fenton and Haber-Weiss reaction. Fenton Weiss reaction is a reaction between ferrous ion and hydrogen peroxide which produces highly reactive hydroxyl radicals implicated in many diseases (Llyod *et al*., 1997). Metal ion-chelating activity of an antioxidant molecule prevents oxy-radical generation and

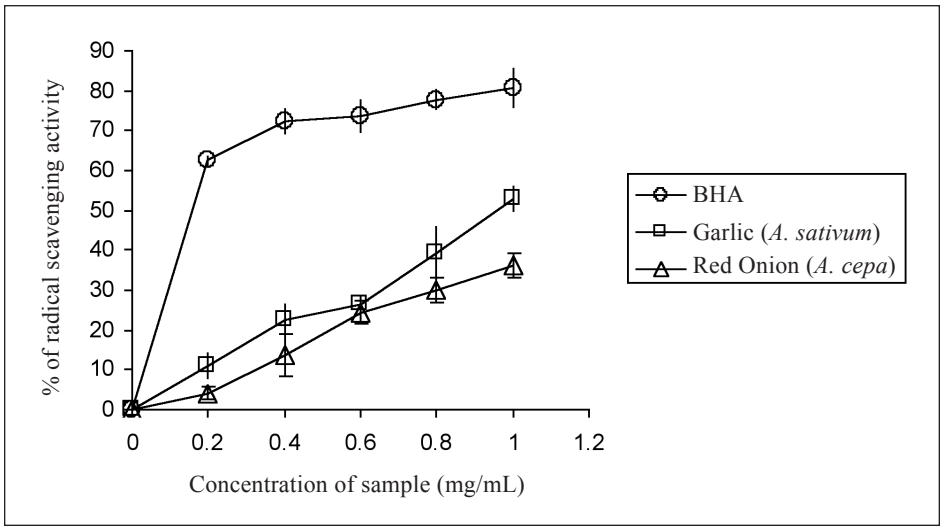

Fig. 2: Comparison of the free radical scavenging activity between the positive control - BHA and samples. IC_{50} value (in mg/mL) for each sample was derived from the graph at 50% free radical scavenging activity

the consequent oxidative damage (Kumar *et al*., 2008). Metal ion-chelating capacity plays a significant role in antioxidant mechanism since it reduces the concentration of the catalysing transition metal in lipid oxidation.

Fig. 3 shows that BHA, garlic and red onion have not much difference in the chelating effects, whereby all of them reached up to 43.14 ± 1.07%, 43.29 ± 3.89%, and 45.00 ± 1.73% at the highest sample concentration of 1.0 mg/mL, respectively. The results also indicate that the ferrous ion chelating effects of red onion are higher than BHA over the increasing concentrations. Furthermore, among the two *Allium* species, the chelating activity of red onion is slightly higher that that of garlic. However, EDTA which serves as the positive control shows the highest percentage of the chelating effect (97.9 ±0.07%). Besides, red onion can be considered as moderate metal chelator since its activities are approximately two times lesser than EDTA. Overall, the results suggest that both *Allium* species may be regarded incapable of strongly obstructing the generation of •OH radicals from Fenton reaction (Kosem *et al*., 2007).

The Relationship between the Results of TPC, DPPH and FIC Assays

There are positive relationships between TPC assay and DPPH radical scavenging activity assay, based on the findings of several previous studies (e.g. Ordoňez *et al*., 2005; Luther *et al*., 2007; Silva *et al*., 2007; Tawaha *et al*., 2007). Most of the researches have mentioned that high phenolic content will lead to high radical scavenging activity. Nonetheless, the present study on garlic and red onion shows a negative relationship between results of TPC assay and DPPH radical scavenging activity assay. Garlic is proven to be better radical scavenger as compared to red onion even though it expresses lower phenolic content. As for FIC assay, most of the previous findings have discovered that even though certain samples possess potent radical scavenging activity, the samples either possess moderate or weak ion chelating activity. For instance, a study conducted by Lim *et al*. (2006) on guava has shown that the samples of guava possess potent radical scavenging activity but they have weak ion chelating effects. Likewise, the current study revealed that garlic and red onion had weak (i.e. for red onion) to

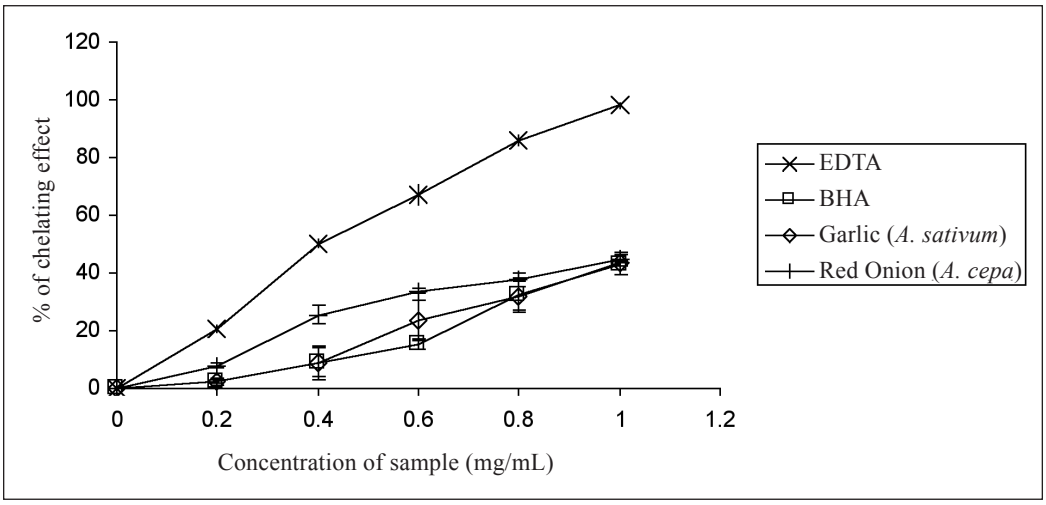

Fig. 3: Comparison of ferrous ion chelating effects between EDTA, BHA and the samples

moderate (i.e. for garlic) ion chelating activities. Overall, the results of the present study do not show good relationship between TPC assay, DPPH radical scavenging activity assay and FIC assay.

CONCLUSIONS

The findings of the current study have shown that red onion (*A. cepa* L.) possesses higher TPC than garlic (*A. sativum* L.*)*. However, garlic has expressed higher free radical scavenging effect (i.e. the primary antioxidant activity) as compared to red onion. As for the ion chelating effect (i.e. the secondary antioxidant activity) measured by the FIC assay, both *Allium* species have been found to have weak (i.e. for red onion) to moderate (i.e. for garlic) ion chelating activities compared to the controls - BHA and EDTA. Overall, the current findings reveal a negative relationship between the results of TPC assay, DPPH radical scavenging activity assay and FIC assay (Table 1). Nonetheless, in order to gain better views on the antioxidant levels and activities in red onion and garlic, further studies on purification, identification and quantification of each phenolic compound and other non-phenolic compounds are necessary in future.

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TABLE 1
Summary of the antioxidant properties for garlic (*Allium sativum* L.) and red onion (*Allium cepa* L.)

Sample	Total phenolic content	Free radical scavenging activity	Metal ion chelating effect
Garlic	37.60 ±2.31 mg GAE/100g	Lower than BHA	Slightly higher than BHA, lower than EDTA
Red onion	53.43 ±1.72 mg GAE/100g	Lower than BHA	Higher than BHA, lower than EDTA

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Phylogenetic Relationships among Different Breeds of Domestic Chickens in Selected Areas of Peninsular Malaysia Using RAPD Markers

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ABSTRACT

The present investigation was carried out to determine the phylogenetic relationships among different breeds of domestic chickens in selected areas of Peninsular Malaysia. Four RAPD primers were used to characterise the genetic similarity among twenty-seven chicken samples from Peninsular Malaysia. Phylogenetic analysis, using neighbour-joining (NJ), generally produced six clusters which were completely distinguished based on the locality of the chickens. A comparatively high level of genetic variation was observed among the village chickens from different locations. A high level of genetic variation was also observed between commercial broiler chicken from selected localities in Peninsular Malaysia and between the commercial broiler and layer chicken in Peninsular Malaysia. Hence, the results indicated the effectiveness of RAPD to detect similarity between chicken lines and their applicability in establishing genetic relationships among chicken populations.

Keywords: Phylogenetic, Random Amplified Polymorphic DNA (RAPD), chickens

INTRODUCTION

Over the last decade, molecular markers have been used to distinguish the different species of *G. gallus*. Prior to the introduction of biochemical and molecular markers, the jungle fowl species were distinguished by morphology and metric quantitative traits (Moiseveya *et al.*, 2003). Based on the archaeological discoveries, there are two hypotheses of the origins of chicken domestication, with one proposing a monophyletic origin and the other, multiple origins from several *Gallus* subspecies (Crawford 1990). In Malaysia, the chickens are basically divided into two main categories, namely village chickens and commercial chickens, which consist of commercial broiler and layer. In particular, village chicken (*kampung* chicken) is a type of chicken that is characterized by medium or low productivity in terms of egg production, egg size, growth, survivability, and inefficient utilisation of feed as compared to commercial broiler. Village chicken is often maintained in a small population in rural areas, such as village, forest, plantation of tropical and subtropical countries and it is not confined in cage (Ramanov & Weigend, 2003). Meanwhile, in the category of commercial chickens, the commercial layer is a type of chicken that is bred for egg production under strong artificial selection (Pirany *et al.,* 2007). Both groups of broilers and layers are maintained under strong artificial selection (Pirany *et al.,* 2007).

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Currently, the breeding schemes for commercial chickens are focused on specialised production lines, derived by an intense selection from a few breeds and very large populations, with a great genetic uniformity of traits under selection (Notter, 1999). Intense selection is mainly concentrated on one or a few traits and thus the approach to homozygosity at same loci may be rapid (Dunnington & Siegel, 1994). As a population approaches homozygosity, the ability to respond to further selection may be impaired. Theoretically, the intense selection may reduce or even exhaust the genetic variability in such populations, thereby reducing progress or rendering further selection ineffective. In poultry breeding, therefore, it is important for the poultry industry to evaluate the levels of genetic diversity in elite pure stocks of the village and commercial chickens (Dunnington & Siegel, 1994; Moiseyeva *et al.*, 2003).

The RAPD markers have particularly been used for genetic and molecular studies, as it is a simple and rapid method used to determine the genetic diversity and similarity in various organisms (Salem *et al.*, 2005). Another interesting characteristic of the RAPD markers is that no prior knowledge of the genome under research is necessary (Fischer *et al.*, 2000; Klinbunga *et al.,* 2000). Meanwhile, the RAPD analysis has extensively been used for genome mapping in chickens (Levin *et al.*, 1993; 1994; Cheng *et al*., 1995), genetic characterisation of highly inbred chicken lines (Potsky *et al*., 1995), identification of specific markers (Zhang *et al*., 1995), genetic diversity, and parentage analysis in chickens and turkeys (Smith *et al.*, 1996), estimation of genetic diversity in inter and intra populations of poultry breeds (Sharma *et al.*, 2001), estimation of genetic diversity of Chinese native chicken breeds (Zhang *et al.*, 2002), genetic similarity between pure lines of chickens in Turkey (Okumus & Kaya, 2005), as well as evaluation of genetic variability and distances among five Iranian native chicken populations (Dehghanzadeh *et al.*, 2009). Currently, the chicken genetic map contains about 1,965 loci within 50 linkage groups and it is estimated that the entire chicken genome contains about 4,000 centimorgans (cM). According to Salem *et al.* (2005), about 235 of these loci have homology with known human or mammalian genes, while the remaining loci are anonymous molecular DNA markers. Sharma *et al.* (2001) have shown the effectiveness of the RAPD in detecting polymorphisms between chicken populations and in establishing the genetic relationship among the chicken populations. Ali & Ahmed (2001) and Ali *et al.* (2003) have demonstrated the potential use of the RAPD analysis for a wide range of applications in poultry breeding. Thus, the author attempted to establish the genetic relationships among different breeds of domestic chickens in selected areas of Peninsular Malaysia using the RAPDs markers.

METHODS

Sample Collection and DNA Extraction

A total of 27 samples were collected from several locations in Peninsular Malaysia (Table 1). The total genomic DNA was extracted from the quill of the feathers using the Wizard Genomic® DNA Purification Kit. The DNA concentration and its purity were examined using the spectrophotometric analysis, based on 260 and 280 nm absorbance and agarose gel electrophoresis analysis.

Random Amplified Polymorphic DNA – Polymerase Chain Reaction (RAPD-PCR)

The RAPD-PCR reaction mixture (25 µL) consisted of 13.3 µL of dH*2*O, 2.5 µL of 10x *Taq* DNA buffer with $(NH_4)_2SO_4$, 2.5 µL of 2.5 mM dNTPs, 2.5 µL of 10 µM of RAPD primer (5'-CGC TGT CGC C-3', 5'-AGT CCT CGC C- 3', 5'-TGG TGG ACC A-3', 5'- GAA TGC GAC G-3'), 0.2 µL of 5U/ µL *Taq* polymerase, 2.0 µL of 25mM $MgCl_2$ and 2.0 µL of 100 ng/ µL DNA template. The final reaction mixture was placed into a thermal cycle (Eppendorf). The RAPD-PCR profile included an initial denaturation step at 94°C for 2 min, followed by 45 cycles at 94°C of 30 sec for DNA denaturation, annealing step at optimised temperature for 30 sec, extension at 72°C for 30 sec and the final extension at

TABLE 1
Sample coding, type of chicken, gender, and locality for each individual

Sample coding	Chicken type	Gender	Locality
VJY1	Village	Male	Pulau Pinang
VJY2	Village	Male	Pulau Pinang
VJY3	Village	Male	Pulau Pinang
VJY4	Village	Male	Pulau Pinang
VJY5	Village	Male	Pulau Pinang
BJY6	Broiler (Cobb)	Male	Johor
VJY7	Village	Female	Perak
VJY8	Village	Female	Perak
BJY9	Broiler (Cobb)	Male	Perak
VJY10	Village	Male	Perak
VJY11	Village	Male	Perak
VJY12	Village	Male	Perak
LJY13	Layer (Hisex)	Female	Perak
VJY14	Village	Male	Perak
VJY15	Village	Male	Johor
BTK16	Broiler (Ross)	Female	Pahang
BTK17	Broiler (Ross)	Female	Pahang
BTK18	Broiler (Ross)	Female	Pahang
BTK19	Broiler (Ross)	Female	Pahang
VTK20	Village	Female	Selangor
VTK21	Village	Female	Selangor
VTK22	Village	Male	Selangor
VTK23	Village	Male	Selangor
VTK24	Village	Male	Selangor
VM25	Village	Male	Kuala Lumpur
VM26	Village	Male	Kuala Lumpur
VM27	Village	Male	Kuala Lumpur

72°C for 10 min were carried out. Meanwhile, the electrophoresis was carried out at 80 V for 110 min. The amplified DNA patterns were analysed on a 2.5% agarose gel electrophoreses and stained with ethidium bromide. The amplified DNA patterns were visualised on a UV transilluminator and photographed using the gel documentation system.

RAPD Data Analysis

The first step in the data analysis was to examine the band patterns of all the DNA samples that were compared with each primer. The presence or absence of the band in each RAPD pattern was recorded using the RAPDistance 1.04 (Armstrong *et al.*, 1994). The presence of the band within each RAPD pattern was scored as one, while the absence of band was scored as zero, in which they were used to estimate the genetic variability within, as well as between the populations. The genetic distances among the different breeds of domestic chickens in selected areas of Peninsular Malaysia were determined according to the Jaccard algorithm. The RAPD distance matrix was used to construct the dendogram of the neighbour-joining (NJ) tree. For each RAPD primer, the genetic similarities among the different breeds of domestic chickens in selected areas of Peninsular Malaysia were determined according to Jaccard similarity coefficient (Rao *et al*., 1996; Romesburg, 2004).

Then, the average of genetic similarity among the different breeds of domestic chickens in the selected areas of Peninsular Malaysia was determined.

RESULTS AND DISCUSSION

Four RAPD primers were screened and yielded distinct polymorphic RAPD profiles in six populations with a total of 104 of polymorphic band patterns and one monomorphic band pattern. These amplified band patterns were found to range from 180 bp to 1530 bp. As expected, the genetic distances and the genetic similarity were the lowest within the intra population and were the highest within the inter population. Furthermore, the genetic distances and genetic similarity were the lowest within similar breed types and were the highest within the dissimilarity breed types.

From the dendogram (*Fig. 1*) that was generated based on the RAPD-PCR fingerprints, the Malaysian village, layer and broiler chicken breeds were clustered into six groups which completely distinguished the locality of each chicken. The first, second and third groups were formed mainly by the village chickens taken from Pulau Pinang, Selangor and Kuala Lumpur, respectively. In this study, high levels of genetic variation were observed among the Malaysian village chickens from various localities. In fact, only the village chickens from the same locality demonstrated low level of genetic variation. The major reason that could have led to the high level of genetic variation among the Malaysian village chickens was the natural selection or breeding. Hence, a high level of genetic variation among the Malaysian village chickens indicates that there is a high level of genetic diversity among the village chickens in Malaysia.

Based on the dendogram, the village chickens from Perak were clustered into Group 4, Group 5, and Group 6. The village chickens taken from Perak and Johor were grouped together in Group 4, whereas the village chickens and the commercial broiler chickens from Perak were grouped together with the commercial broiler chicken from Johor in Group 6. As observed within Group 5, the village chickens and the commercial layer chicken from Perak were grouped with the commercial broiler chickens from Pahang. This grouping was probably due to the import of the commercial chickens between the states in Peninsular Malaysia. In counterpoint to the village chickens, the broiler chickens from Pahang and a commercial layer chickens from Perak were clustered together within Group 5 (*Fig. 1*). The classification of the Malaysian commercial broiler and layer chickens was in contrast to a previous study conducted by Shen *et al.* (2002), whereby the Japanese Chunky broilers and commercial layers were grouped into two different groups.

In this study, a high level of genetic variation observed among the broiler chickens in Peninsular Malaysia was found to be with the finding of Shen *et al.* (2002), whereby a high level of genetic variation was observed among the Japanese Chunky broilers. Nevertheless, the finding of this study is inconsistent with the previous studies conducted by Sharma *et al.* (2001) and Ali *et al.* (2003). According to Sharma *et al.* (2001), a low level of genetic variation (genetic dissimilarity) was observed among the commercial broilers. In addition, Ali *et al.* (2003) also reported that a high level of genetic similarity was observed among the commercial broiler chickens from different localities. One of the reasons that could have led to the high level of genetic variation among the broiler chickens was the different breeds of broiler chickens were used for breeding strategies. Similarly in this study, a high level of genetic variation was also observed between the commercial broiler and layer chickens in Malaysia. Zhang *et al.* (2002) reported that a great difference of genetic variation was observed between the broiler and layer chicken breeds. Hence, the finding of this study is compatible with the study by Zhang *et al.* (2002). Nevertheless, this study was found to be inconsistent with the study conducted by Ali *et al.* (2003) who observed a high level of genetic similarity between the commercial broiler and layer chickens from different localities. The

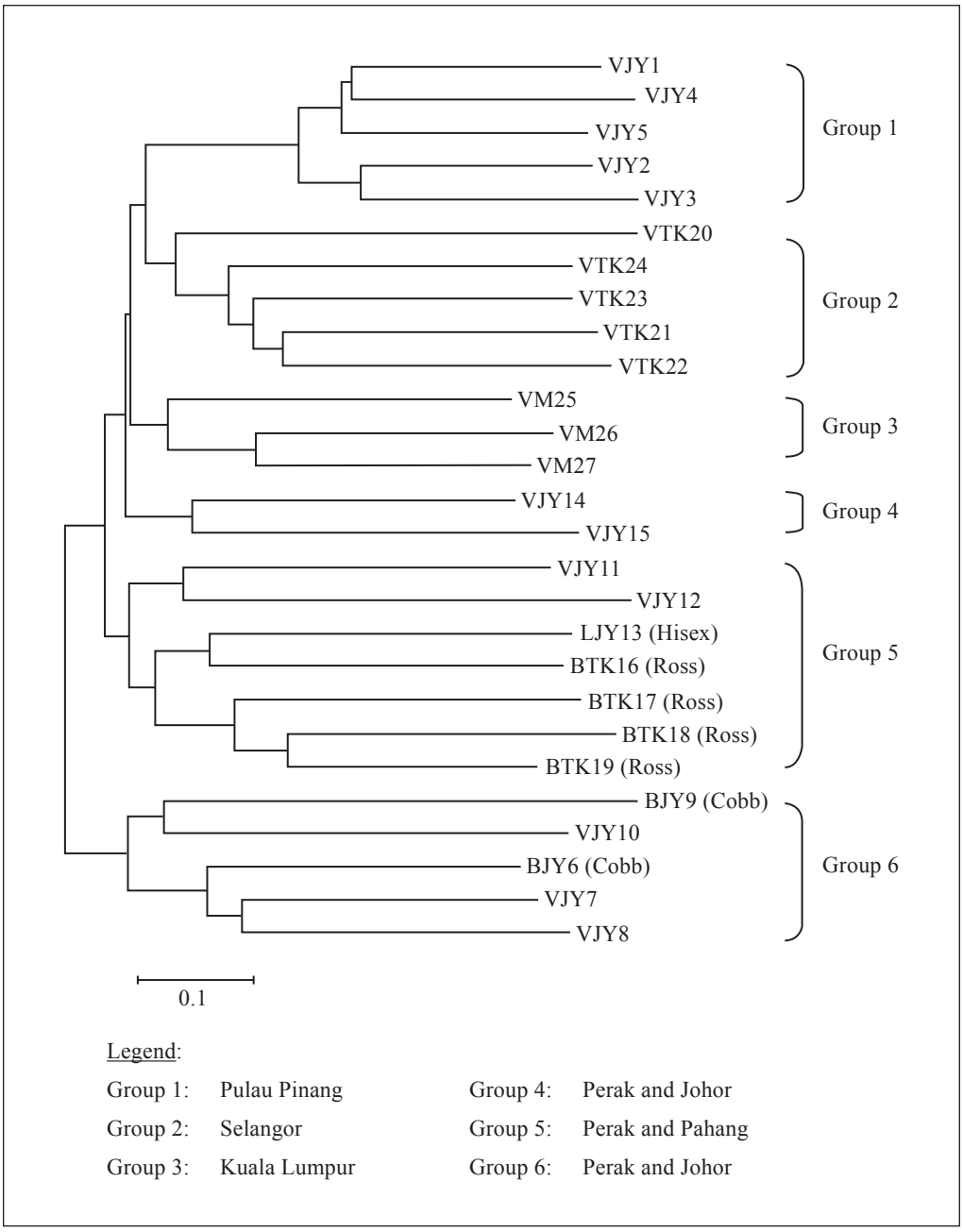

Fig. 1: The dendogram of different breeds of domestic chickens from the selected areas in Peninsular Malaysia generated based on the RAPD-PCR finger print patterns using four RAPD primers

reasons that could have led to the high level of genetic variation among the commercial broiler and layer chicken were the different breeds of chicken, whereby the broilers were bred for meat production and the commercial layers were bred for egg production (Sharma *et al.*, 2001; Shen *et al.*, 2002).

Another finding of this study is that high level of genetic variation was observed between the Malaysian village and commercial (broiler and layer) chickens. As compared to the study by Sharma *et al.* (2001), a high level of genetic variation was observed among the native chicken breed (Kadaknath) and the exotic chicken breeds in India. Furthermore, Zhang *et al.* (2002) revealed that the genetic variation between the Chinese native chicken breeds and commercial layer chickens was relatively high based on the RAPD analysis. Hence, this study is in agreement with the studies of Sharma *et al.* (2001) and Zhang *et al.* (2002).

The estimation of the genetic variability in populations is a critical prerequisite for optimising breeding strategies and regulating germplasm conservation which requires routine detection and monitoring methods (Sharma *et al*., 2001). Apart from the different breeds of village chickens and commercial chickens, one of the reasons that could have led to the high level of genetic variation among the domestic breeds was the natural and artificial selections, whereby the selection (natural or artificial) could have altered the allele frequency by reducing the likelihood that one or more genotypes would contribute to the next generation (Delany, 2003). However, if the poultry breeders continue to use population selection without taking the genetic selection schemes into consideration, the genetic variability in such population might become exhausted or even cause undesirable correlated responses to occur (Nordskog, 1965; Siegel & Dunnington, 1997). Hence, the maintenance of the genetic variation and genetic diversity among the Malaysian village chickens and commercial chickens is a crucial step to protect the Malaysian chickens against the present and unforeseen threats of avian disease. In addition, the knowledge of genetic similarity between the populations is prerequisite for establishing crossbreeding systems (Sharma *et al*., 2001). In this study, it has proven that the RAPD can provide an efficient system in determining the genetic diversity and genetic similarity of chickens due to its simplicity, speed and low cost. Moreover, Sharma *et al.* (2001) revealed the effectiveness and reliability of the RAPDs as a potential genetic marker in determining the genetic diversity and genetic similarity due to the common bands with very high degree of sequence homology, and this thus may be allelic to each other.

CONCLUSION

The present results demonstrated the efficacy of the RAPD markers in detecting polymorphism and establishing genetic relationships among the chicken breeds (Cobb, Ross, Hisex, and village chickens) in Peninsular Malaysia. Moreover, the present results demonstrated the ability of the RAPD markers to distinguish the locality of chickens from Peninsular Malaysia**.**

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Effect of Seminal Plasma Removal, Washing Solutions, and Centrifugation Regimes on Boer Goat Semen Cryopreservation

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ABSTRACT

Three experiments were carried out to improve semen quality during cryopreservation process. Total motility, forward motility, acrosome integrity, live spermatozoa, and normal spermatozoa were measured as semen quality. In Experiment 1, the effects of seminal plasma removal were analyzed by using two different extenders (GE and FE). The removal of seminal plasma gave higher and significant ($P<0.05$) effect in the total motility, forward motility, and live spermatozoa after cryopreservation. For two different extenders, however, the differences were not observed on the semen quality. In Experiment 2, three different washing solutions (namely, phosphate buffered saline, normal saline and Tris-based extender) were tested to evaluate the effects of semen quality after cryopreservation. Tris-based extender (TCG) conferred the highest ($P<0.05$) sperm quality values in the total motility, forward motility, and live spermatozoa after cryopreservation. In Experiment 3, the effects of different centrifugation regimes (3000 × g for 3 min, 1600 × g for 10 min, 800 × g for 15 min) were evaluated on Boer semen quality. Semen quality parameters (namely, total motility, forward motility, acrosome integrity, and live spermatozoa) were significantly ($P<0.05$) higher for cryopreserved spermatozoa centrifuged with 3000 × g for 3 min than the others. In conclusion, the removal of seminal plasma, washing solution TCG, and the use short-term centrifugation with a relative high g-force could contribute to the increased Boer semen quality after cryopreservation.

Keywords: Boer goat, centrifugation, cryopreservation, seminal plasma, washing solution

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INTRODUCTION

Semen cryopreservation is essential for the application of reproductive techniques such as AI and IVF which contribute to increase production of goat and genetic selection schemes (Leboeuf *et al*., 2000). However, cryopreservation causes ultrastructural, biochemical, and functional damages on spermatozoa due to the temperature changes resulting in decreased motility and viability. In addition, causes of reduced sperm motility are related to seminal plasma enzymes. Therefore, seminal plasma plays an important role in sperm survival during cryopreservation process (Salamon and Ritar, 1982).

The deterioration and toxic effect of the seminal plasma were observed when goat's semen was diluted with egg yolk or milk extender. Nowadays, these extenders are widely used for the frozen storage of small ruminant semen (Salamon & Maxwell, 2000). The presence of enzymes (bulbourethral secretion glycoprotein-60 and egg yolk coagulating enzyme) in the seminal plasma caused the harmful interactions between seminal plasma and egg yolk or milk (Nunes *et al*., 1982, Leboeuf *et al*., 2000). Meanwhile, bulbourethral secretion glycoprotein-60 (BUSgp60) has a triacylglycerol hydrolase activity which decreases sperm motility and movement quality by disruption of cell membrane (Pellicer-Rubio & Combarnous, 1998). Phospholipase A_2 activity of egg yolk coagulating enzyme (EYCE) catalyse the hydrolysis of egg yolk phosphatidycholine (PC) into fatty acids and lysophophatidycholine (LPC). LPC has toxic effect on buck spermatozoa by acting like a detergent on biomembrane, resulting in a loss of motility, membrane integrity and consequently low fertility rate (Upreti *et al*., 1999).

Therefore, several researchers reported that the removal of seminal plasma had favourable effect on semen freezing and thawing properties in buck (Ritar & Salamon, 1982; Corteel, 1992; Kozdrowski *et al*., 2007). However, Tuli & Holtz (1994), Azeredo *et al*. (2001) and Peterson *et al*. (2007) observed no favourable effect of the removal of seminal plasma in buck semen cryopreservation. Sariozkan *et al*. (2010) also described that a high fertility rate, with or without centrifugation/washing, buck semen could be achieved with the Bioxcell extender. Therefore, the benefits of seminal plasma removal presented in literature are quite variable until now (Purdy, 2006).

Removal of seminal plasma is a time consuming process that can damage cells if it is performed improperly; however, if it is done correctly, it can be beneficial (Purdy, 2006). Therefore, some previous research have used a variety of washing solutions and concentration regimes to find out the proper method for the improvement of cryopreserved semen quality. The centrifugation regimes and washing solutions used in buck semen cryopreservation were 800 x g for 15 min with Tris citric acid buffer (Tuli & Holtz, 1994), 600 × g for 10 min with Krebs-Ringer phosphate plus sodium citrate (Azeredo *et al*., 2001), 1200 × g for 15 min with Tris citric acid glucose (TCG) buffer (Peterson *et al*., 2007), 1500 × g for 10 min with TCG (Kozdrowski *et al*., 2007), and 1000 × g for 10 min with Ringer's latate (Sariozkan *et al*., 2010). In other species, several researchers have studied the effects of centrifugations regimes. Among other, Carvajal *et al*. (2004) observed the influence of different centrifugation regimes (400, 800, 1600, and 2400 × g) and reported that the use of short-term centrifugation with a relative high g-force (2400 × g for 3 min) caused a positive effect on Boar sperm cryosurvival. Webb & Dean (2009) described that post thaw motility of frozen stallion sperm was not different between centrifugation treatments (700 × g for 15 min, 600 × g for 12 min, and 400 × g for 7 min). Nonetheless, studies evaluating the effects of washing solutions and centrifugation regime on Boer goat semen cryopreservation are still very limited. Moreover, the results presented in the literature are quite variable. Hence, to overcome this variability, the researchers should endeavour to reach a consensus that generally addresses accepted practices for the effects of seminal plasma and centrifugation in Boer goat semen cryopreservation. Therefore, these experiments were carried out to analyze the effects of seminal

plasma removal using two different extenders, three different washing solutions and different centrifugation regimes on the characteristics of Boer goat semen before freezing and after thawing.

MATERIALS AND METHODS

Animals, Semen Collection and Evaluation

Semen samples from five mature Boer goats (2 and 4 yr of age) were used in this study. Ejaculates were collected twice a week from the Boer goats with the aid of an artificial vagina. Immediately after collection, the ejaculates were immersed in warm water bath at 37°C and semen assessment was performed within approximately 20 min. Only ejaculates between 1 and 2 ml in volume, spermatozoa with >70% progressive motility, and a concentration of higher than 2.5 x 10^9 spermatozoa per straw were used for the freezing process. A total of 30 ejaculates were used in this study.

The volume of each ejaculate was measured in a graduated test tube and consistency was subjectively scored (0 to 3). The mass activity of the semen was measured on a score of 0 to 5. The sperm concentration of each ejaculate was determined by means of a haemocytometer. Live and dead spermatozoa, as well as morphologically normal spermatozoa percentages were assessed using nigrosin-eosin stain (Evan & Maxwell, 1987). The percentage of acrosome integrity (with normal apical ridge) was determined by evaluating sperm smears which were stained with nigrosin-eosin and examined under phase contrast microscope at x1000 magnification under oil immersion objective with bright light (Yildiz *et al*., 2000). Spermatozoa (200 cells per slide) in duplicate for each treatment were assessed as well. The percentage of the motility of spermatozoa in each specimen was evaluated under a phase contrast microscope at ×200 magnification by placing a 5 µl drop of diluted semen on a slide covered with a glass cover slip (22 mm × 22 mm) from three selected representative fields subjectively. The mean of the three successive estimations was recorded as the final motility score. S perm motility was assessed by modifying the category of the WHO laboratory manual (WHO, 2002).

Extenders

Two types of semen extenders were used to analyse the effects of seminal plasma removal on Boer goat semen cryopreservation (Foote, 1970; Liu *et al*., 1998).

Glucose based extender (GE) - the cooling extender consisted of 250 mM Tris, 88.5 mM citric acid, 70 mM glucose, 18% egg yolk (v/v) and antibiotics (5000 IU penicillin, 5 mg streptomycin, 10 mg neomycin per ml). The freezing extenders were composed of 250 mM Tris, 88.5 mM citric acid, 18% egg yolk (v/v) and 8% glycerol (v/v).

Fructose-based extender (FE) - the cooling extender consisted of 312.53 mM Tris, 110 mM citric acids, 55 mM fructose, 20% egg yolk (v/v) and antibiotics (5000 IU penicillin, 5 mg streptomycin, 10 mg neomycin per ml). The freezing extender was composted of 312.53 mM tris, 110 mM citric acid, 55 mM fructose, 20% egg yolk (v/v) and 6.8% glycerol (v/v).

Glucose based extender (GE) was used to evaluate the effects of washing solutions and centrifugation regimes. All the chemicals were reagent grade and were purchased from Sigma-Aldrich, St. Louis, MO.

Experimental Designs

A total of three experiments were conducted in this study. The first experiment was replicated three times from each of the four Boer goats. Following the initial evaluation, the ejaculates were equally divided into two parts. One part was diluted with normal saline at the ratio of (1:1 v/v) and subjected to centrifuge at 1500 x g for 5 min for the removal of seminal plasma. The washed spermatozoa was split again into two equal aliquots and immediately extended with two different extenders. The other part of the ejaculate was also divided into two aliquots, but without the removal of seminal plasma and diluted with the two extenders (GE and FE), respectively.

In Experiment 2, the study was replicated two times from each of five Boer goats. Immediately after the initial evaluation, the ejaculated semen was equally divided into three aliquots and diluted with each of the three different washing solutions (phosphate-buffered saline [PBS], normal saline [NS], Tris-based extender [TCG], cooling extender of GE without egg yolk at the ratio of (1:1 v/v) (O'Meara *et al*., 2007; Evan & Maxwell, 1987; Kozdrowski *et al*., 2007). Meanwhile, the seminal plasma was removed by centrifugation at 1500 × g for 5 min.

Experiment 3 was replicated two times from each of the four Boer goats. Following the initial evaluation, the semen was mixed with cooling extender of GE without egg yolk at the ratio of (1:1 v/v) and equally divided into three parts. Diluted spermatozoa were centrifuged by using three different centrifugation regimes (3000 × g for 3 min, 1600 × g for 10 min, and 800 × g for 15 min) and all of supernatants were discarded.

Cooling, Freezing and Thawing of Spermatozoa

After the removal of seminal plasma, the semen was diluted with cooling extender. Semen dilution procedure was carried out by using two step dilution methods. The diluted semen was incubated at room temperature (26°C) for 10 min. The extended semen were subsequently placed in a cooling chamber at 5°C and maintained for 2.5 hr. The extended semen was then diluted in the freezing extender to obtain the final concentration of 150×10^6 spermatozoa per straw and kept for 30 min. After that, the sperm suspension was loaded into 0.25 ml straws. The total cooling time lasted for about three hours. The straws were horizontally placed on an aluminium rack and frozen in liquid nitrogen vapour, about 5 cm above the surface of liquid nitrogen for 7 min, and then immersed into the liquid nitrogen for storage. After 2 days, the thawing procedure was carried out in a water bath (37°C) for 30 sec. Immediately after thawing, the total motility, forward motility, acrosome integrity, percentage of live spermatozoa, and normal spermatozoa were evaluated.

Statistical Analysis

The data were analysed using the SPSS software system (Version 12.0, SPSS, Chicago, IL). The sperm quality parameters in Experiment 1 were analysed using a 2-way factorial analysis of variance. The values were expressed as the mean ± the standard error of the mean (S.E.M) and the level of statistical significance was considered as $P<0.05$. In Experiments 2 and 3, the means were analyzed using a one-way analysis of variance, followed by the Tukey's post-hoc test to determine the significant differences in all the semen quality parameters between the groups.

RESULTS

The removal of seminal plasma had a significant influence on semen quality parameters before freezing and after thawing (Table 1). Washing of spermatozoa provided significantly ($P<0.05$) higher effect on progressive motility and live spermatozoa before freezing. After thawing, washed spermatozoa were found to have significant ($P<0.05$) effects on the total motility, forward motility, acrosome integrity and the percentage of live spermatozoa. However, the percentages of normal spermatozoa were not affected by washing spermatozoa. No significant differences ($P>0.05$) were observed between the two different extenders in all the semen quality parameters, but GE conferred a better effect in Boer goat semen cryopreservation.

Meanwhile, different washing solutions (PBS, NS, and TCG) had significant ($P<0.05$) effects on the semen quality parameters (Table 2). In particular, TCG washing solution conferred the highest sperm quality values in progressive motility and live spermatozoa before freezing. Acrosome integrity and normal spermatozoa, on the contrary, were not significantly ($P>0.05$) affected by different washing solutions before freezing, but higher values were found in TCG. After thawing, washing solution TCG was found to have the highest significant effect ($P<0.05$) on

TABLE 1
The effects of seminal plasma removal using two extenders on the semen characteristics of Boer goat

Before freezing	Progressive motility %	Intact acrosome %	Live spermatozoa %	Normal spermatozoa %
SP removed (FE)	74.58 ± 1.86	81.17 ± 1.25	76.58 ± 1.52	95.50 ± 0.53
SP removed (GE)	77.50 ± 1.69	80.50 ± 1.81	75.67 ± 2.06	95.83 ± 0.52
SP retained (FE)	69.58 ± 2.42	77.83 ± 1.66	71.83 ± 1.80	96.33 ± 0.58
Sp retained (GE)	70.83 ± 2.03	78.67 ± 1.47	72.08 ± 1.90	96.92 ± 0.69

After thawing	Total motility %	Forward motility %	Intact acrosome %	Live spermatozoa %	Normal spermatozoa %
SP removed (FE)	61.67 ± 2.41 ab	52.08 ± 2.08 ac	66.67 ± 1.92 ab	61.08 ± 2.20 ab	88.42 ± 1.22
SP removed (GE)	64.17 ± 2.59 b	55.83 ± 2.37 a	70.25 ± 1.95 b	64.08 ± 2.12 b	89.25 ± 1.57
SP retained (FE)	55.83 ± 2.45 a	43.33 ± 2.33 b	63.92 ± 1.65 a	57.08 ± 2.05 a	90.50 ± 1.35
Sp retained (GE)	58.33 ± 2.49 a	47.08 ± 2.08 bc	64.50 ± 2.31 a	58.33 ± 2.19 a	91.08 ± 1.21

Means within column with different alphabetical superscripts (a,b,c) are significantly different at least as P<0.05 (by LSD). Mean ± SEM of the semen of three ejaculates obtained from four individuals are shown. SP=seminal plasma, FE=fructose based extender, GE=glucose based extender

TABLE 2
The effects of three different washing solutions on Boer goat semen characteristics

Before freezing	Progressive motility %	Intact acrosome %	Live spermatozoa %	Normal spermatozoa %
PBS	79.50 ± 1.17^{a}	85.30 ± 1.32	78.70 ± 1.45^{a}	93.40 ± 0.81
NS	76.50 ± 1.50^{b}	84.20 ± 1.16	78.90 ± 1.27^{b}	91.40 ± 0.60
TCG	84.50 ± 0.89^{c}	87.50 ± 1.43	83.20 ± 1.45^{c}	95.60 ± 0.58

After thawing	Total motility %	Forward motility %	Intact acrosome %	Live spermatozoa %	Normal spermatozoa %
PBS	54.50 ± 2.29^{a}	47.50 ± 1.11^{a}	67.70 ± 0.78^{a}	61.40 ± 0.95^{a}	88.70 ± 1.51
NS	51.50 ± 2.89^{b}	44.50 ± 1.74^{b}	66.10 ± 1.19^{a}	59.60 ± 1.31^{a}	87.90 ± 1.50
TCG	61.50 ± 2.24^{c}	54.50 ± 0.89^{c}	73.90 ± 0.66^{b}	68.50 ± 0.86^{b}	89.90 ± 0.89

Mean ± SEM within each column, means with different alphabetical superscripts (a,b,c) are significantly different at least as P<0.05 (ANOVA – Post hoc test). PBS = Phosphate Buffer Solution, NS = Normal Saline, TCG = Tris citric acid glucose extender

all the semen quality parameters, except for the normal spermatozoa.

Centrifugation regimes had a significant influence on the semen quality of Boer goat, before freezing and after thawing (Tables 3). Before freezing, centrifugation regime C1 (3000 × g for 3 min) showed significantly (P<0.05) higher effect in progressive motility than the others. However, there were no significant differences in intact acrosome, live spermatozoa and normal spermatozoa between C1 (1600 × g for 10 min) and C2 (800 × g for 15 min) before freezing. After thawing, the semen quality parameters (total motility, forward motility, acrosome integrity, and live spermatozoa) were significantly (P<0.05) higher for frozen-thawed spermatozoa centrifuged with C1 than for the others. The differences between the spermatozoa centrifuged with C2 and C3 were not significant for the semen quality parameters forward motility, intact acrosome, live spermatozoa and normal spermatozoa. Nonetheless, C2 gave a higher significant (P<0.05) effect in the total motility and better sperm quality values than C3 in the Boer goat semen cryopreservation.

TABLE 3
The effects of different centrifugation regimes on the semen characteristics of Boer goat

Before freezing	Progressive motility %	Intact acrosome %	Live spermatozoa %	Normal spermatozoa %
C1	73.75 ± 1.83[a]	79.50 ± 1.46	73.88 ± 2.40[a]	91.25 ± 1.64
C2	66.88 ± 2.30[b]	76.63 ± 1.73	70.75 ± 2.19[a]	92.63 ± 1.31
C3	62.50 ± 3.13[b]	75.13 ± 1.98	68.13 ± 1.96[b]	94.13 ± 0.93

After thawing	Total motility %	Forward motility %	Intact acrosome %	Live spermatozoa %	Normal spermatozoa %
C1	60.62 ± 2.74[a]	50.63 ± 2.74[a]	68.38 ± 2.73[a]	63.38 ± 2.69[a]	86.75 ± 2.05
C2	53.12 ± 2.30[b]	44.37 ± 2.74[b]	63.88 ± 2.64[b]	56.50 ± 2.69[b]	87.75 ± 1.89
C3	48.75 ± 1.83[c]	43.75 ± 2.27[b]	61.25 ± 0.66[b]	55.25 ± 2.43[b]	89.38 ± 1.75

Mean ± SEM within each column, means with different alphabetical superscripts (a,b,c) are significantly different at least as $P<0.05$ (ANOVA – Post hoc test). C1 = 3000 x g for 3 min, C2 = 1600 × g for 10 min, C3 = 800 × g for 15 min

DISCUSSION

The removal of seminal plasma increased Boer semen quality in cryopreservation process. This is in agreement with the findings of Ritar & Salamon (1982), Machado & Simplicio (1992), Love *et al*. (2005) and Kozdrowski *et al*. (2007) who reported a beneficial effect of removing seminal plasma on the freezeability of semen. This, however, contradicts with the findings of Tuli & Holtz (1994), Gil *et al*. (2000), Azeredo *et al*. (2001) and Peterson *et al*. (2007) who reported that the removal of seminal plasma decreased motility in frozen-thawed spermatozoa. Furthermore, Angora buck sperm frozen, with or without centrifugation/ washing in the Bioxcell extender, demonstrated higher percentages of subjective motility (58.1 ± 3.0% and 53.5 ± 3.8%, respectively) compared to that of the groups Tris extender (40.9 ± 1.8% with centrifugation and 45.0 ± 3.1% without centrifugation) (Sariozkan *et al*., 2010). Some of these contraindications may be attributed to several factors, such as season, different processing procedures (namely, washing solution, centrifugation regimes, cooling and freezing rate), and the concentration of seminal plasma remaining after centrifugation. Moreover, species, breeds and individual variation are also critical factors because the compositions of seminal plasma and sperm membrane vary greatly between species and individuals. Among other, seasonal variation is one of the most important considerations (Leboeuf *et al*., 2000).

Seminal plasma cholesterol concentration, which was most probably related to the use of extracellular lipids for the protection of sperm membrane integrity vary between seasons (Beer-Ljubic *et al*., 2009). Furthermore, seasonal differences influence the type of proteins found in seminal plasma. The absence of critical proteins, such as 20-kDa (Perez-Pe *et al*., 2001), 25-kDa (Lessard *et al*., 2000), 26-kDa (Gerena *et al*., 1998) caused lower recovering effect on sperm viability during non-breeding season. These proteins are found only during the breeding season. Major proteins of bovine seminal plasma bind to the low-density lipoprotein fraction of hen's egg yolk leading to adhesion of these proteins to sperm membrane and prevent cryodamage (Manjunath *et al*., 2002). However, there was a negative correlation between one of the protein bands (16 kDa) and semen freezability due to the impaired linking of egg yolk lipoproteins to sperm membrane (Brandon *et al*., 1999; Zahn *et al*., 2006). Meanwhile, vesicular gland solutions, which only occur in the breeding season, partially inhibit the negative effect of BUSgp-60 bulbourethral gland secretion (Nunes *et al*., 1982). EYCE hydrolyses egg yolk phosphatidylcholine (PC) and produces lysophosphatidylcholine (LPC) which has toxic effects by acting on biomembranes of spermatozoa as a detergent. The formation and

strength of detergent properties are temperature dependent (Peterson *et al*., 2007). Therefore, the amount of compositions formed in the seminal plasma and the strength of detrimental effect to the spermatozoa during cryopreservation process is dependent on the type of weather or type of season.

Meanwhile, washing solution TCG increased semen quality during cryopreservation process. It seems that the addition of glucose into the washing solution causes better ability to support energy utilisation for goat sperm cryosurvival. This result is supported by Waite *et al*. (2008) who found that the centrifugation media INRA 96 containing proteins gave more superior effect in equine sperm motility than HELL centrifugation media. Cochran *et al*. (1984) have reported that the effect of centrifugation on equine spermatozoa motility is influenced by the type of extender used prior to or following centrifugation. Therefore, this experiment further confirms that washing solution could influence sperm quality parameters after cryopreservation.

Centrifugation at 3000 × g for 3 min markedly improved the quality of Boer semen cryopreservation. Hence, the use of high g-force and short-term centrifugation is clearly recommended in the centrifugation step of Boer goat sperm. Carvajal *et al*. (2004) confirmed the current results by reporting that the use of short-term centrifugation with a relative high g-force (2400 x g for 3 min) showed significantly higher on boar sperm cryosurvival and oocyte penetration ability. Moreover, Shekarriz *et al*. (1995) have also reported that the time of centrifugation is more critical than g-force for inducing human sperm damage in the preparation of sperm for assisted reproductive techniques. Although centrifugation causes potential damage to the spermatozoa, the use of high g-force centrifugation could enhance to remove the ejaculate contaminants, such as abnormal and dead spermatozoa. Dead spermatozoa produced reactive oxygen species (ROS) which have harmful effect on spermatozoa during cryopreservation (Upreti *et al*., 1999). Therefore, the use of high g-force centrifugation could lead to increase in the percentage of high quality spermatozoa. Based on the criteria adopted in these studies, washing solution and centrifugation regimes could be stated to have significantly influenced the buck semen cryosurvival. Washing solution TCG and high g-force centrifugation with short duration caused better improvement in Boer goat semen quality after cryopreservation.

In conclusion, this study has indicated that the practical and beneficial effects can be obtained by removing seminal plasma through centrifugation (3000 × g for 3 min) with TCG washing solution in Boer goat semen cryopreservation protocol. However, these results are based only on motility characteristics, and therefore, further fertility trials are required as the ultimate test of improved cryopreservation.

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Phylogenetic Analysis of the Malaysian *Rhinolopus* and *Hipposideros* Inferred from Partial Mitochondrial DNA Cytochrome *b* Gene Sequences

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ABSTRACT

The phylogenetic relationships among 10 species of *Rhinolophus* and 10 species of *Hipposideros* from Borneo and Peninsular Malaysia were successfully inferred from the partial mitochondrial DNA (mtDNA) cytochrome (cyt) *b* sequences. Of the 413 nucleotide positions examined, there were 171 positions (41.4%), of which 164 positions (95.9%) were parsimoniously informative. The phylogenetic trees reconstruction using neighbour-joining (NJ), unweighted maximum parsimony (MP) and maximum likelihood (ML) methods suggest the monophyletic clustering of these families. The interspecific relationships within Rhinolophidae were completely resolved, while those within Hipposideridae were not fully resolved, as supported by the low bootstrap values. Overall, the phylogenetic analysis using partial mtDNA cyt *b* gene was useful to discriminate these complicated taxa and successfully revealed the misidentification of several specimens before due to their similar morphologies.

Keywords: Cytochrome *b*, *Hipposideros*, mitochondrial DNA, phylogenetics, *Rhinolophus*

INTRODUCTION

Rhinolophus (Horseshoe bats) and *Hipposideros* (Roundleaf bats) are widely distributed throughout the tropic, sub-tropic and temperate zones of the Old World region (Corbet & Hill, 1992; Feldhamer *et al*., 1999; Hutson *et al*., 2001). In Malaysia, there are currently 22 *Rhinolophus* species recorded with 18 species found in Peninsular Malaysia and 11 species in Borneo. Generally, the rhinolophids are small to medium in size, having an elaborate complex noseleaf and a raised portion called sella that is very useful for identification among the species of this genus (Payne *et al*., 1985; Corbet & Hill, 1992). The ears are sorted from moderate to large sized with a moderate long tail that is completely enclosed within their interfemoral membrane (Payne *et al*., 1985; Vaughan, 1986; Corbet & Hill, 1992).

On the other hand, 17 *Hipposideros* species are currently recorded, in which 16 species are distributed in Peninsular Malaysia and 10 species are found in Borneo (Payne *et al*., 1985; Corbet & Hill, 1992; Khan, 1992; Koopman, 1994). Varying from small to moderate large in size with no sella, the hipposiderids have an elaborate noseleaf with a horse-shoe shaped anterior leaf while the posterior leaf is low and rounded that is divided into several pockets by vertical septa (Payne *et al*., 1985; Corbet & Hill, 1992; Francis, 2001). Their ears range from moderate small to large size with a low antitragus, having very

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small eyes and the tail is short to moderate long, which is completely enclosed in the interfemoral membrane (Payne *et al*., 1985; Corbet & Hill, 1992).

The rhinolopids and the hipposiderids are generally found roosting in caves, tunnels, buildings, hollow trees and foliage including rock crevices recorded mostly from the tall forest understorey (Payne *et al*., 1985; Corbet & Hill, 1992). The closed association between the two families has led to many arguments in their classification and grouping. Some authors, including Vaughan (1986), Findley (1993), Wilson & Reeder (1993) and Koopman (1994), had classified both *Rhinolophus* and *Hipposideros* into a single family of Rhinolophidae alone, while Corbet & Hill (1992), Hutson *et al*. (2001) and Simmons (2005) had grouped these genera separately into each distinct family of Rhinolophidae and Hipposideridae.

On top of that, there is a lack of current information on the taxonomic and phylogenetic relationships of Rhinolophidae and Hipposideridae, particularly in Malaysia. A similar study was done by Wang *et al*. (2003), but using only the specimens from China with mostly different target species. A preliminary study by Besar *et al*. (2005) successfully revealed the phylogenetic relationships of only five Bornean *Rhinolophus* species. Paul (2007), however, was unable to resolve the interspecific relationships of *Hipposideros* in Borneo, using the combined 12S and 16S mtDNA sequences, due to unstable phylogenies supported by low bootstraps values.

Therefore, there is a need to evaluate the interspecific and intraspecific relationships of Rhinolophidae and Hipposideridae in Malaysia, as well as to examine the monophyletic status of these two families. *Nycteris tragata* (family: Nycteridae) and *Megaderma spasma* (family: Megadermatidae) were used as the outgroups as they are classified together with these two families in the superfamily of Rhinolophoidea. This is to reveal the relationship at the family level for both Rhinolophidae and Hipposideridae.

MATERIALS AND METHOD

Tissue samples were collected from 10 species of *Rhinolophus* and 10 species of *Hipposideros*, including other representatives from *Nycteris tragata* and *Megaderma spasma* as the outgroups. The bats were collected using standard mist-nets and four-bank harp traps (Mohd-Azlan *et al*., 2004). The selected bats were euthanised using chloroform and tissue samples were preserved in 95% ethanol. Some additional samples were taken from alcohol preserved specimens from Universiti Malaysia Sarawak (UNIMAS) Zoological Museum and the Department of Wildlife and National Park (DWNP) Museum. The DNA extractions of the tissues samples were made following Grewe *et al*. (1993) and the amplification was done in polymerase chain reaction (PCR) *et al*using a pair of cyt *b* primer; GludG-L: 5'-TGACTTGAARAACCAYCGTTG-3' and CB2-H: 5'- CCTCAGAATGATATTTGTCC TCA-3' (Palumbi *et al*., 1991).

A total reaction volume of 25µl comprising of 2.5 µl 10× PCR buffer was used, 1.5 µl magnesium chloride (25mM), 0.5 µl dNTP (10mM), 1.25 µl of each forward and reverse primers (10µM), 15.8 µl deionised water, 2.0 µl DNA template and 0.2 µl *Taq* DNA polymerase. The amplification process included initial denaturation at 93°C for 2 minutes, denaturation for 30 cycles at 93°C for 1 minute, primer annealing at 56°C for 1 minute, polymerase extension at 72°C for 2 minutes and a final extension at 72°C for 5 minutes. The PCR products were loaded into 1% agarose gel containing ethidium bromide and run for about 45 minutes at 90V. Fragment sizes of the amplified products were estimated to be between 400 bp to 500 bp length using a low range DNA ladder (100 bp). The PCR products were then purified using a purification Kit following the protocol provided by the manufacturer (Fermentas), purposely to remove any trace of contamination that might be present in the PCR products including salt, PCR reagents and primer-dimer before being sent to a private laboratory (First Base Sdn. Bhd.) for DNA sequencing. Only

the forward strands (GludG-L) were sequenced using the ABI ® 377 DNA automated sequencer with the ABI PRISM BigDye® Terminator version 3.0 Cycle Sequencing Kit.

All the sequences were uploaded into GenBank and each specimen has been provided with accession number for future revisions (EF095237 and EF108140 to EF108177)and were aligned using the Clustal X 1.81 program (Thompson *et al*., 1997) and saved in clustal and nexus formats. The nucleotide compositions and genetic pairwise distances among the examined species were calculated using the Kimura 2-parameter model (Kimura, 1980), whereas the phylogenetic relationships of the species were analysed using the Phylogenetic Analysis Using Parsimony (PAUP*) program version 4.0 beta (Swofford, 1998) and constructed using the neighbour-joining (NJ), unweighted maximum parsimony (MP) and maximum likelihood (ML) methods.

The NJ clustering was performed using the Kimura 2-parameter evolution model (Kimura, 1980) and the MP method was conducted using full heuristic search while ML analysis corresponded to the Hasegawa, Kishiro and Yano of HKY85 evolutionary model (Hasegawa *et al*., 1985). All trees were rooted with *N. tragata* and *M. spasma* as the outgroups. Phylogenetic confidence was estimated by bootstrapping (Felsenstein, 1985) with 1000 replications of data sets for both NJ and MP, whereas for ML, 100 replications (Hedges, 1992) of data sets were applied.

According to Miyamoto & Boyle (1989) and Irwin *et al*. (1991), the transversion substitutions in mammals showed a linear relationship with time. The estimation of divergence (Saitou & Nei, 1987) between the ingroups (Rhinolophidae and Hipposideridae) and the outgroups (Nycteridae and Megadermatidae) of the mtDNA cyt *b* gene was calculated using a constant transversion rate of 0.2% per million years ago (mya) (Bastian *et al*., 2001), where each species was grouped into their respective families and the rate calculated were multiplied by 0.2%.

RESULTS

Eighty five specimens from 10 *Rhinolophus* species, 10 *Hipposideros* species, *N. tragata* and *M. spasma* (Table 1) were successfully sequenced and analysed, together with a sequence of *H. armiger* (AF451332), where the sequences began at 7 bp until 419 bp of the 1140 bp complete mtDNA cyt *b* sequence (*H. armiger*; DQ297585) representing 36.2% of the total cyt *b* gene sequence.

Of the total 413 bp length nucleotide sequences analysed, 171 positions (41.4%) were variable, in which 164 positions (95.9%) of the variable sites were parsimoniously informative. The nucleotide translation into a total of 137 amino acid sequences produced 25 variable positions (18.25%), in which 24 positions (96.00%) of the variable sites were parsimoniously informative. The empirical base compositions of the mtDNA cyt *b* among the examined species were T (26.8%), C (31.1%), A (27.6%) and G (14.5%). The frequencies of T and A (54.4%) were slightly higher than those of C and G (45.6%), resulting in anti-G bias sequenced, which is a characteristic for the mitochondrial gene (Cantatore *et al*., 1994; Briolay *et al*., 1998).

Meanwhile, the genetic pairwise distances calculated using the model of Kimura 2-parameter among the species of *Rhinolophus*, *Hipposideros*, *Nycteris*, and *Megaderma* are shown in Table 2. Generally, the percentage of genetic distance within the genus *Rhinolophus* ranged from 3.7% (between *R. luctus* and *R. trifoliatus*) to 14.2% (between *R. sedulus* and *R. acuminatus*), with a mean of 9.5% genetic distance. In *Hipposideros*, the percentage of genetic distance ranged from 7.0% (between *H. armiger* and *H. larvatus*) to 16.5% (between *H. bicolor* and *H. cervinus; H. bicolor* and *H. coxi*), with a mean of 12.0% genetic distance.

Overall, the average genetic distance between *Rhinolophus* and *Hipposideros* was 17.0%. The average genetic distance between *Rhinolophus* and *Nycteris* was 22.1% and that between *Hipposideros* and *Nycteris* was 20.8% respectively, whereas the average genetic

TABLE 1
Scientific and local name of rhinolophid and hipposiderid species, sample collection, sample size and GenBank accession numbers used in the study

		Species	Common name	Sample collection			n	Genbank accession no.
				Swk	Sbh	PM		
Rhinolophidae	***Rhinolophus***	*R. acuminatus*	Acuminate horseshoe bat		√		2	EF108154, EF108155
		R. affinis	Intermediate horseshoe bat	√		√	9	EF108156 to EF108160
		R. borneensis	Bornean horseshoe bat	√			3	EF108161, EF108162
		R. creaghi	Creagh's horseshoe bat		√		2	EF108163, EF108164
		R. luctus	Great woolly horseshoe bat	√		√	5	EF108165, EF108166
		R. philippinensis	Philippine horseshoe bat	√	√		5	EF108167 to EF108169
		R. pusillus	Least horseshoe bat	√			2	EF108170, EF108171
		R. sedulus	Lesser woolly horseshoe bat	√		√	5	EF108172 to EF108174
		R. stheno	Lesser brown horseshoe bat			√	1	EF108175
		R. trifoliatus	Trefoil horseshoe bat	√		√	4	EF108176, EF108177
Hipposideridae	***Hipposideros***	*H. armiger*	Great roundleaf bat				1	AF451332
		H. ater	Dusky roundleaf bat	√			3	EF108139, EF108140
		H. bicolor	Bicolored roundleaf bat	√		√	6	EF108142, EF108143
		H. cervinus	Fawn roundleaf bat	√			4	EF108141, EF108144, EF108146
		H. cineraceus	Ashy roundleaf bat	√		√	5	In progress.
		H. coxi	Cox's roundleaf bat	√			5	EF108145, EF108147, EF108148
		H. diadema	Diadem roundleaf bat	√			4	EF108149
		H. dyacorum	Dayak roundleaf bat	√			4	EF108150, EF108151
		H. galeritus	Cantor's roundleaf bat	√			5	In progress.
		H. larvatus	Intermediate roundleaf bat	√			3	EF108152, EF108153
		H. ridleyi	Ridley's roundleaf bat	√			4	EF095237
Outgroups								
		Nycteris tragata	Hollow-faced bat	√		√	2	In progress.
		Megaderma spasma	Lesser false vampire	√			2	In progress.
						Total	86	

Swk = Sarawak, Sbh = Sabah, PM = Peninsular Malaysia, n = number of samples

distance between *Rhinolophus* and *Megaderma* was 15.1% and that between *Hipposideros* and *Megaderma* was 16.2%, respectively. Additionally, the estimation on the times of divergence is shown in the NJ topology (*Fig. 1*). Meanwhile, the divergence times between Rhinolophidae and Hipposideridae was predicted at around 31.5 mya ± 4.5 mya.

Phylogenetic tree constructions using the NJ (*Fig. 1*), MP (*Fig. 2*), and ML (*Fig. 3*) methods suggested that Rhinolophidae and Hipposideridae formed their own monophyletic group, with relatively low to moderate bootstrap support (68% in NJ; 73% in MP; 80% in ML), although the groupings of the examined species in both the families were slightly different where the arrangements were similar, as inferred by the different methods.

In NJ (*Fig. 1*), Rhinolophidae was divided into four sub-groups; where Group 1 consisted of

TABLE 2
Genetic pairwise distance (%) among 10 *Rhinolophus* species, 10 *Hipposideros* species, *N. tragata* and *M. spasma* analysed in this study based on mtDNA cyt b gene sequences. Distances were calculated using the Kimura 2-parameter model (Kimura, 1980)

Species	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]	[19]	[20]	[21]	[22]	[23]
[1] *R. acuminatus*																							
[2] *R. affinis*	10.5																						
[3] *R. borneensis*	12.2	11																					
[4] *R. creaghi*	8.6	7.7	11.8																				
[5] *R. luctus*	12	11.7	12.1	10.6																			
[6] *R. philippinensis*	10.6	10.5	6.8	10.5	12.2																		
[7] *R. pusillus*	10.8	11.2	5.6	9.4	11.5	3.9																	
[8] *R. sedulus*	14.2	12.4	11.8	12.5	7.1	12.1	11.7																
[9] *R. stheno*	8.3	7.1	11.5	4.6	11.8	11.1	10.3	12.6															
[10] *R. trifoliatus*	12.1	10.8	11.9	11.1	3.7	12.4	11.3	7.7	12.3														
[11] *H. armiger*	14.9	14.8	19.6	13.7	16.1	16.4	15.7	16.6	13.6	17.2													
[12] *H. ater*	17.2	19	21.8	18.4	17.9	18.9	18.9	20.4	16.9	18.7	13.6												
[13] *H. bicolor*	16.8	17.9	18.8	18	19.8	18.3	17.3	20.4	16.5	19.2	14.5	10.7											
[14] *H. cervinus*	16.3	16.2	18	14.5	17.1	17.1	16.2	17.9	13.9	17	14.4	16.8	16.5										
[15] *H. cineraceus*	16.6	17.5	17.8	15.6	16.3	15.7	15.1	17.1	14	17.3	12.7	10.8	11	14.4									
[16] *H. coxi*	17.4	18.8	19.5	16.2	15.5	18	17.6	18.2	16.3	15.7	14.8	14.4	16.5	13.8	16.2								
[17] *H. diadema*	17.5	17.7	17.7	16.2	16.6	16.4	15.1	17.4	16.2	18.1	10.5	12.8	15.1	13.6	11.5	15.9							
[18] *H. dyacorum*	14.8	18.1	19.3	15.7	17.4	17.3	16.1	19.2	15.3	17.9	11.9	9.6	10.3	12.1	8.8	14.4	9.8						
[19] *H. galeritus*	14	14.9	16.9	15.4	17.3	14.8	13.8	15.7	13.5	16.6	13	13.9	14.3	11	12.2	14.2	12.4	11.3					
[20] *H. larvatus*	13.1	13.1	16	14.4	15	14.2	14.2	15.9	13	15.4	7	13.5	13.8	14.3	13.5	16.1	10.9	12.2	12.7				
[21] *H. ridleyi*	14.7	16.7	18.5	16.6	16.3	16.4	15.8	17.7	13.7	17.2	14.1	8.8	10.5	13.9	9.4	15.4	11.2	8.5	12.6	13.5			
[22] *N. tragata*	21.2	22.4	23.8	20.3	21.1	22.7	22	22.3	19.5	22.3	22.3	21.1	21.2	19.8	21.9	21.4	21.2	18.9	18.9	21.3	21.9		
[23] *M. spasma*	16.1	14.1	16.3	13.6	15.3	15.2	13.4	15.7	13.2	16.3	14.9	17.2	16.9	16.3	15	17.6	16.4	15.4	15.3	13.6	17.7	17.4	

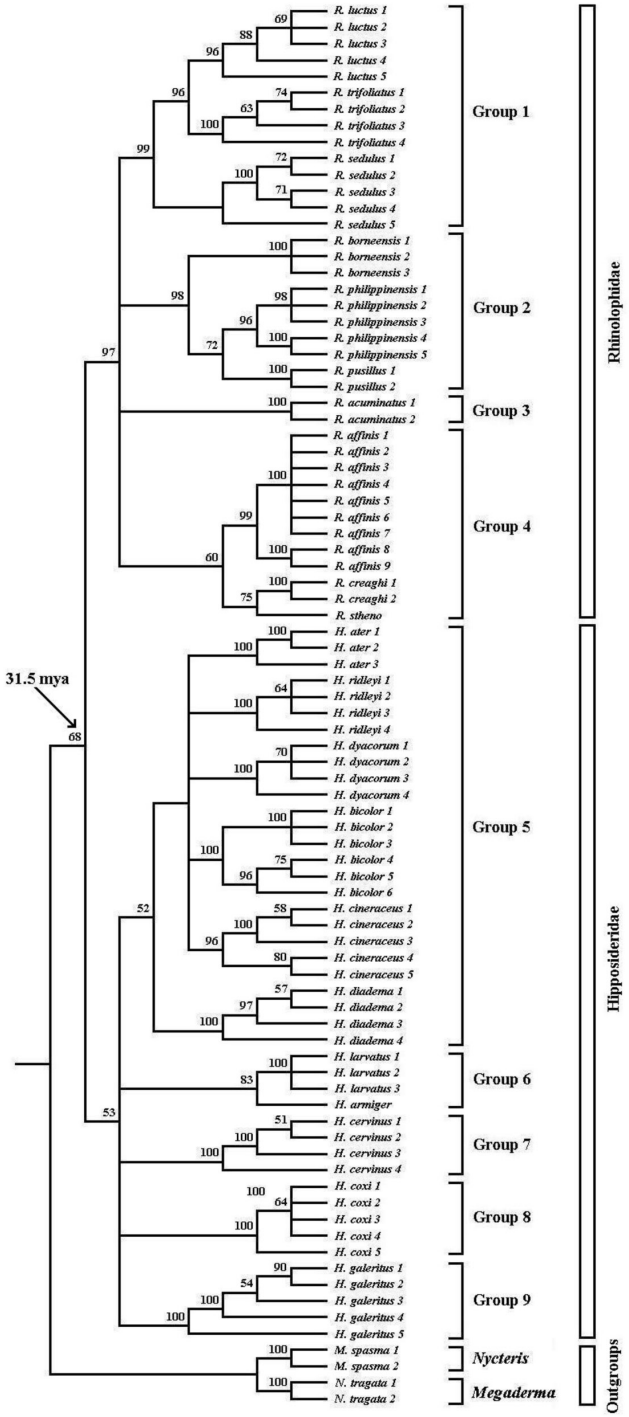

Fig. 1: Phylogentic relationships of rhinolophids *and* hipposiderids *under study based on 413 mtDNA cyt* b *gene sequences. The phylogeny is a single tree recovered using NJ analysis. Values on the branches represent NJ bootstrap estimates, based on 1000 replicates. Only bootstrap values >50% are shown*

R. luctus, R. trifoliatus and *R. sedulus*, Group 2 consisted of *R. borneensis, R. philippinensis* and *R. pusillus*, Group 3 consisted of *R. acuminatus* alone and Group 4 consisted of *R. affinis, R. creaghi*, and *R. stheno*. The clustering result within this family was supported by 97% of the bootstrap values. In Hipposideridae, five sub-groups were identified, in which, *H. ater, H. ridleyi, H. dyacorum, H. bicolor, H. cineraceus*, and *H. diadema* were clustered in Group 5, whereas *H. larvatus* and *H. armiger* were categorized in Group 6, *H. cervinus* in Group 7, *H. coxi* in Group 8, and *H. galeritus* in Group 9. However, the grouping within Hipposideridae was only supported by 53% of the bootstrap values.

Using the MP analysis with the unweighted characters, the tree was 673 bp with a consistency index (CI) of 0.3507 and a retention index (RI) of 0.8544 (*Fig. 2*). The phylogeny and branching within Rhinolophidae was similar to the NJ clustering, supported by 84% of the bootstrap values. Within Hipposideridae, four sub-groups were obtained. The fifth group was formed by *H. ater, H. ridleyi, H. dyacorum, H. bicolor* and *H. cineraceus*, while the sixth group was formed by *H. diadema, H. larvatus* and *H. armiger*, the seventh group comprised of *H. cervinus* and *H. coxi* and the eighth group consisted of *H. galeritus* alone. Similarly, the clustering within this family was supported by only 55% of the bootstrap values.

Using the ML procedure (-Ln likelihood = 1461.22137) (*Fig. 3*), the groupings within Rhinolophidae (83% of bootstrap value) were similar to those obtained using the NJ and MP. The ML analysis, however, produced different groupings within Hipposideridae (75% of the bootstrap value), in which three sub-groups were formed. Group 5 consisted of *H. ater, H. ridleyi, H. dyacorum, H. bicolor* and *H. cineraceus*, whereas Group 6 was represented by *H. diadema, H. larvatus* and *H. armiger*, and the remaining species of *H. cervinus, H. coxi* and *H. galeritus* were clustered together in Group 7.

DISCUSSION

All the phylogenies of NJ, MP and ML methods, inferred from 413 bp of the mtDNA cyt *b* gene, resulted in the monophyletic clustering of *Rhinolophus* and *Hipposideros*. The genetic pairwise distances obtained from the present study were also comparable to those of Wang *et al*. (2003) in classifying the two groups into different families. The separation of Rhinolophus and Hipposideros was further supported by the allozyme variability (Maree & Grant, 1997).

Other molecular results revealed that the Rhinolophidae and Hipposideridae are sister taxa, as reported by Maree & Grant (1997), Jones *et al*. (2002), Teeling *et al*. (2002; 2005), Guillén *et al*. (2003) and Gunnell & Simmons (2005). Similar results were also obtained through karyotypical and morphological analyses presented by Bogdanowicz & Owen (1992; 1998), as well as Hand & Kirsch (1998). However, the present study was unable to reveal the ancestral lineage of the family, as they were supported by only moderate bootstrap values which might be due to the short sequence length analysed, small sample sizes, as well as incomplete representatives of the whole Malaysian rhinolophids and hipposiderids, respectively.

According to Hand *et al*. (1994), the oldest bat fossils of Rhinolophidae were recorded from the late Oligocene-early Miocene in Lake Eyre Basin, Australia. Guillén *et al*. (2003) reported that the bat fossils of Hipposideridae had been recorded from the Oligocene of Africa and the Miocene of Africa, Australia and South East Asia. In the present study, Rhinolophidae and Hipposideridae were predicted to have diverged from each other around 31.5 mya ± 4.5 mya, whereas Guillén *et al*. (2003) estimated the event happened approximately 35 mya. Guillén *et al*. (2003) also suggested that the origin of the *Rhinolophus* species was from Europe, which contradicted with the findings of Bogdanowicz (1992), and Bogdanowicz & Owen (1992), who suggested that the origin of Rhinolophidae was from Asia.

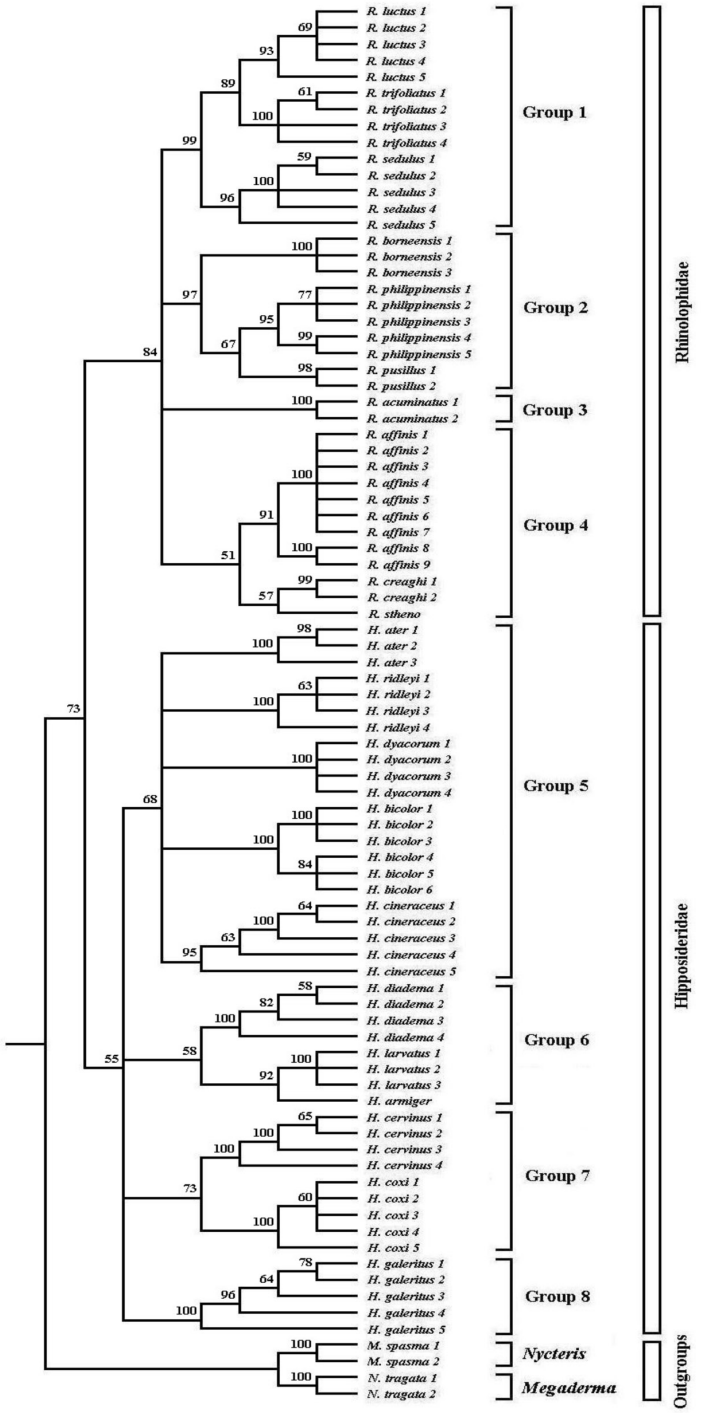

Fig. 2: The unweighted and rooted MP tree based on nucleotide data set of partial mtDNA cyt b *gene (tree length=673; CI=0.3507; RI=0.8544). The values on the branches represented the MP bootstrap estimates, based on 1000 replicates. Only bootstrap values >50% are shown*

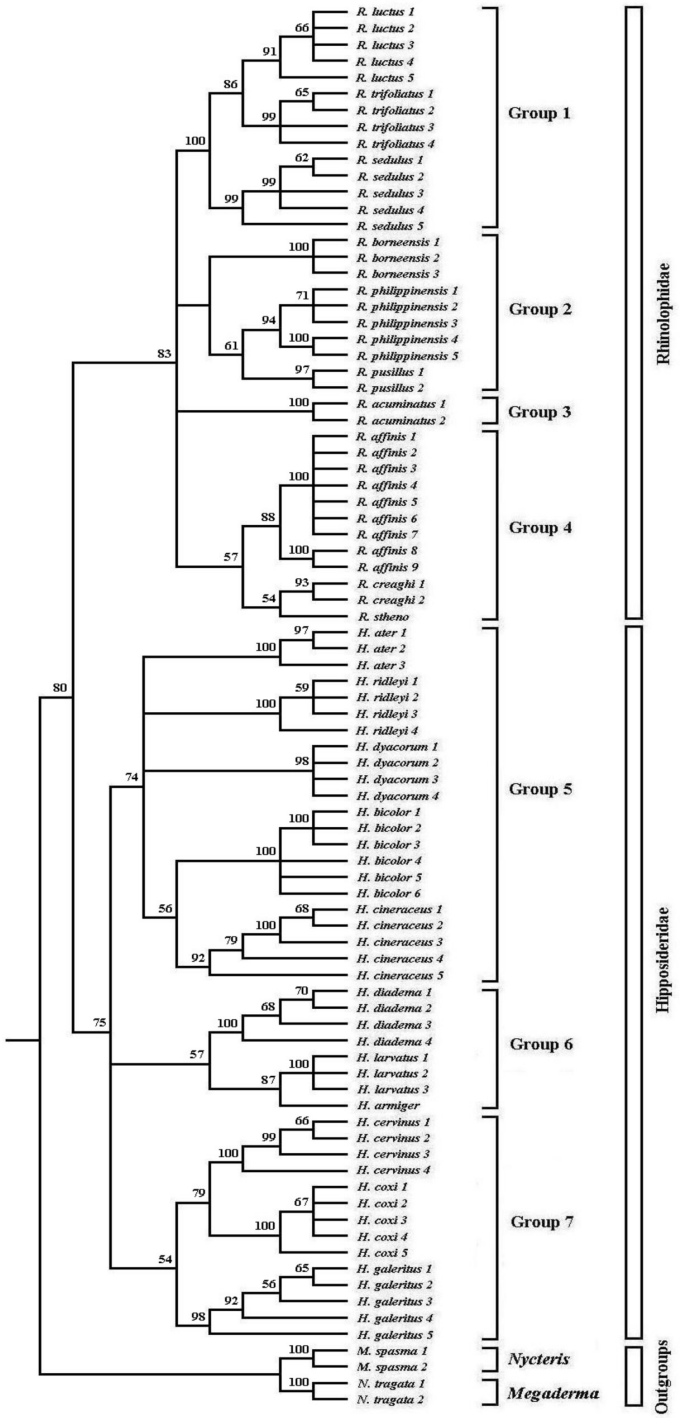

Fig. 3: Rooted ML tree (-Ln likelihood=1461.22137) generated based on the nucleotide data set of partial mtDNA cyt b *gene. Values on the branches represented the ML bootstrap estimates, based on 100 replicates. Only bootstrap values >50% are shown*

In Group 1, the grouping of *R. luctus, R. trifoliatus* and *R. sedulus* showed similar arrangement, as reported in phenetic clustering by Bogdanowicz (1992) and the phylogenetic studies by Bogdanowicz & Owen (1992) and Guillén *et al*. (2003). The arrangement of *R. luctus* and *R. trifoliatus* in a sub-group, which was derived from *R. sedulus*, was fully supported by high bootstrap value. Bogdanowicz & Owen (1992), through their ordination method, had classified these species into the *trifoliatus* group that further assigned it as the sub-genus, *Aquias* by Guillén *et al*. (2003). Meanwhile, Payne *et al*. (1985) stated that this group was morphologically similar with long body fur and the presence of lateral lappets, with *R. luctus* being the easiest species to identify, as they are the largest rhinolophid.

The clustering of *R. borneensis, R. philippinensis*, and *R. pusillus* in Group 2 is congruence to the species grouping proposed as the sub-genus *Rhinophyllotis* by Guillén *et al*. (2003). However, Bogdanowicz (1992) and Bogdanowicz & Owen (1992) placed *R. philippinensis* into its own group, together with *R. marshalli* and *R. macrotis* that were not examined in the present study. According to Payne *et al*. (1985), *R. borneensis* shares similarities of their external morphological characters with *R. pusillus*, but the latter possesses shorter forearm length and has a very small noseleaf. Beside that, *R. philippinensis* is easily distinguished from the other two species by its larger body size.

In addition, Guillén *et al*. (2003) also included *R. acuminatus* as the basal species within this sub-genus. However, the *R. acuminatus* analysed in this study was independently clustered in Group 3, although it was found to be morphologically similar to the other rhinolophid species in Group 4 (Payne *et al*., 1985; Corbet & Hill, 1992). Bogdanowicz (1992) and Bogdanowicz & Owen (1992) also placed this species out of the group consisting of *R. affinis, R. creaghi* and *R. stheno*.

The association between *R. affinis, R. creaghi* and *R. stheno* in Group 4 is similar to Bogdanowicz's (1992) phenogram, Bogdanowicz & Owen (1992) and Guillén *et al*. (2003), who categorised these species into *Coelophyllus* sub-genus, together with the presence of *R. arcuatus*. However, using the robust analysis from the ordination technique, Bogdanowicz (1992) classified *R. affinis* and *R. acuminatus* into the *rouxi* group, where the researcher separated *R. arcuatus, R. creaghi* and *R. stheno* into the *euryotis* group.

From this study, some specimens that were primarily assigned as *R. arcuatus (R. affinis 1, R. affinis 2, R. affinis 3* and *R. affinis* 5) and *R. acuminatus (R. affinis* 6) in the field were misidentified (*Figs. 1*, *2* and *3*) and clustered together into the *R. affinis clade*. They were only differed by a value of less than 2% divergence that further confirmed the recognition of these specimens as the *R. affinis*. Bradley & Baker (2001) noted that a genetic distance of less than 2% in cyt *b* sequences of mammals was typical of population and intraspecific variation.

For the Hipposideridae in the present study, the species grouping proposed by Hill (1963), Corbet & Hill (1992) and Simmons (2005) was not supported, as the members of the *bicolor* group seemed to be paraphyletic, where the species of *H. cervinus, H. coxi*, and *H. galeritus* were clustered in Group 7. This finding was also at variance with the phylogenetic studies of Bogdanowicz & Owen (1998) and Paul (2007), who also used similar species. These discrepancies could have occurred due to the different data implemented by each author. Only the ML analysis was further discussed due to its moderate bootstrap support of 75% that was regarded as sufficiently resolved topology (Huelsenbeck & Hillis, 1993).

The clustering among *H. ater, H. ridleyi, H. dyacorum, H. bicolor*, and *H. cineraceus* in Group 5 were generally supported by Payne *et al*. (1985), Corbet & Hill (1992) and Bogdanowicz & Owen (1998). Generally, these species share similar characters of not possessing lateral leaflets, and having similar facial ornamentation with simple noseleaves (except for *H. ridleyi* that possesses large noseleaf) (Payne *et al*., 1985; Khan, 1992). Moreover, the darker brown noseleaf colour and the pointed ear tips of

H. dyacorum are useful for discriminating this particular species from the others.

In Group 6, the close relationship between *H. larvatus* and *H. armiger* as sister clades was supported, and the phylogeny showed a similar arrangement as that described by previous authors, including Allen (1938) and Wang *et al*. (2003), together with the presence of *H. diadema* in the group (Bogdanowicz & Owen, 1998). In addition, this arrangement showed a similar result to Paul (2007), who used a combination of the 12S and 16S mtDNA sequence.

The grouping of *H. diadema, H. larvatus* and *H. armiger* was also supported by Payne *et al*. (1985), Khan (1992), Koopman (1994) and Kingston *et al*. (2006), as these species have three or more lateral leaflets. However, *H. larvatus* can be easily recognised by the length of the forearm, which ranges from 52 to 65 mm, whereas *H. armiger* (FA: 85-97 mm) and *H. diadema* (FA: 76-87 mm) can be identified using their body coloration (Payne *et al*., 1985; Khan, 1992; Kingston *et al*., 2006).

The remaining species of the bicolor group, including *H. cervinus, H. coxi* and *H. galeritus* were independently clustered in Group 7, although the arrangement was poorly supported with 54% of the bootstrap value. Previously, Hill (1963) had missed several diagnostic characters that were obviously useful in differentiating *H. cervinus* and *H. galeritus*. Later, Jenkins & Hill (1981) revealed that both *H. cervinus* and *H. galeritus* were wrongly classified as the same species due to the absence of several possible characters which might be useful for discriminating these species, as applied by Kitchener *et al*. (1993a,b). This included the details of the nose leaf structure and the measurements of the second phalanx on the third digit.

Both Payne *et al*. (1985) and Corbet & Hill (1992) supported the correlation among *H. cervinus, H. coxi* and *H. galeritus* as these species have two lateral leaflets and a similar facial structure. In addition, *H. cervinus* and *H. galeritus* can be differentiated from each other through the noseleaves structure and tail length, while *H. coxi* possesses darker body coloration and larger, more complex noseleaf than the other two species.

Overall, the findings of this study have shown that the partial mtDNA cyt *b* gene is useful to resolve the interspecific relationships within selected species of Rhinolophidae, but was unsuccessful in completely reviewing the phylogenetic relationships among the selected Malaysian *Hipposideros*.

CONCLUSION AND RECOMMENDATIONS

In conclusion, the phylogenetic relationships inferred from the partial mtDNA cyt *b* gene supported the monophyletic grouping of Rhinolophidae and Hipposideridae, as two different families have provided new information on the limited knowledge regarding the microchiropterans in Malaysia. The taxonomy and systematic of *Rhinolophus* are similar to the metric phenetic clustering shown by Bogdanowicz (1992) and the phylogenetic studies by Bogdanowicz & Owen (1992) and Guillén *et al*. (2003). However, the phylogenetic relationships within *Hipposideros* were incompletely resolved.

It also revealed that the misidentification of specimens in the field was common among closely related species, as the morphological characteristics of some species are similar and overlapping. Thus, correct field identification of species is important in order to infer an accurate biological diversity of the fauna and to avoid incorrect conclusions (Sazali *et al*., 2008). Although the monophyletic status of these families is currently reviewed, further molecular studies should be conducted using larger sample sizes, the complete mtDNA cyt *b* gene (approximately 1140 bp length) or other coding regions (e.g. COI, ND2), including other species, to fully assess the phylogenetic relationships of the horseshoe bats and roundleaf bats. Hopefully, the findings of this research can be applied for effective future management and conservation of these insectivorous bats, particularly in relation to the Malaysian specimens.

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Management Practices Affecting Helminthiasis in Goats

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ABSTRACT

The study was conducted to investigate the effects selected management practices have on worm burden in goats as reflected by faecal egg counts. The faecal examination of six goat farms for the quantitative presence of strongyles was conducted in Terengganu. A questionnaire was developed and directed to six farmers on the management practices adopted on their farms. The management practices selected in the study were grazing time, mineral block supplementation, type of drug used, breed, and source of animal, grass type, additional feed, and drenching personnel. The data analysis was done through systematic approaches using t-test, Spearman correlation and ANOVA. Afternoon grazing reduced the mean FEC nearly five-fold compared to morning grazing and mineral block supplementation reduced FEC two-fold compared to unsupplemented goats ($P<0.05$).

Keywords: Management practices, goats, helminthiasis

INTRODUCTION

Nematode parasites cause high mortalities in small ruminant livestock in Malaysia as in other parts of the tropics, and economic losses are mainly due to subclinical infections (Sani *et al*., 2004). Endoparasitism in small ruminants referring mainly to haemonchosis is acknowledged to be the second most important cause of mortalities in small ruminants in Malaysia. The control of worms in small ruminants in Malaysia, like elsewhere, relies heavily on chemical dewormers or anthelmintic drugs. Anthelmintics being easily available and affordable, together with the government-subsidized ruminant health programme implemented in Malaysia, have led to the emergence of widespread anthelmintic resistance among nematodes in sheep and goats. Hence, the alternatives to chemotherapeutic control of gastrointestinal nematode parasites of small ruminants need to be explored. Manipulations in management practices have been shown to reduce parasitism.

As a means of reducing anthelmintic use, nutritional supplements have been employed to enhance resilience to parasitism and maintain productivity in small ruminants which is frequently lost during subclinical infections (Knox *et al*., 2006). Well-nourished animals are known to withstand the effects of worm infection much better than those given a lower plane of nutrition. Meanwhile, the resistance of animals to establishment of worm larvae can be enhanced by improved protein nutrition (Sykes & Coop,

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2001). Another option of worm control by grazing management using a rotational system based on epidemiological knowledge has been proven to be successful when used consistently. Small ruminants grazing for three to four days in an area which was emptied for five to six weeks has reduced worm burdens (Sani *et al*., 2004).

Therefore, this study was conducted to investigate the effect of management practices adopted by farms on worm burden, as reflected by faecal egg counts. The management practices selected were grazing time, mineral block supplementation, type of drug used, breed, source of animal, grass type, additional feed, and drenching personnel on these farms.

MATERIALS AND METHODS

The study was conducted in the state of Terengganu which is located in the north-eastern part of West Malaysia between May to August 2009, i.e. during a relatively dry season (Department of Meteorology Malaysia, 2010, http://www.met.gov.my/). Terengganu has the biggest population of goats in the country.

Eight smallholder goat farms in the state were examined for their parasitic status by a faecal survey conducted a few weeks before the start of the study. The smallholder farms were defined as having less than 50 goats. Two farms each in three districts in the state were chosen; namely, Marang, Kuala Berang and Setiu with assistance from the Department of Veterinary Services, Terengganu.

The six selected farms were instructed not to drench the animals eight weeks prior to the collection of faecal samples. This measure was done to ensure that the goats had a sufficiently high number of worms as reflected by more than 250 eggs per gram. All the farms practice a semi-intensive production system, where the goats are housed in raised floor barns and allowed to graze for two hours during the day. The goats comprised of Jamnapari, Katjang, Boer, and Jamnapari crosses. Meanwhile, out of a total of 230 goats, 63 were male and the rest were female goats. The ages of the goats ranged from less than 1 year to 5 years.

Data on management practices adopted by the owners of the six farms were obtained from

TABLE 1
Management practices adopted by farms and their mean FEC

Farm no./Management practices	1	2	3	4	5	6
Grazing time	Afternoon	Morning	Morning	Afternoon	Afternoon	Afternoon
Mineral block supplementation	Yes	No	Yes	Yes	Yes	Yes
Drug type	Iver	Mix (Iver and BZ)	Iver	Mixed (Iver, Clos, BZ)	Mixed (Leva, Iver, Bz)	Mixed (Clos, Iver, BZ)
Breed	Jamnapari	Jamnapari	Mixed (Boer, Jamnapari, Katjang)	Mixed (Jamnapari, Boer)	Jamnapari	Mixed (Jamnapari, Katjang)
Source of goats	Local	Indonesia	Indonesia	Local	Local	Local
Type of Grass	Improved	Improved	Improved	Native	Native	Improved
Additional feed	Yes	Yes	Yes	No	Yes	No
Drenching personnel	DVS	Farmer	DVS	Farmer	DVS	Farmer
Mean FEC (S.E.)	769.70 (301.97)	925.00 (175.58)	4291.18 (992.45)	627.50 (140.83)	620.93 (174.69)	2681.58 (442.86)

a survey based on a questionnaire. Meanwhile, data on worm burden were collected based on the faecal egg counts (FEC) using the Modified McMaster technique (Coles *et al*., 1992). FEC is a reliable indicator of worm burden as shown in a study by Israf *et al*. (1996) in which a significant positive correlation of faecal egg count with worm burden was found in caprine gastrointestinal helminthiasis. The correlation was present for FEC and *H. contortus* but there was no correlation with *T. colubriformis* burdens. Therefore, the FEC was used in this study to represent the worm burden as it is not possible to enumerate the worm burden in live goats.

The management practices chosen in this study were grazing time, mineral block supplementation, type of drug used, breed, source of animal, grass type, additional feed, and drenching personnel. As for the grazing time, two categories were involved; namely morning grazing (from 8 am to 10 am) and afternoon grazing (from 4 pm to 6 pm). Mineral block supplementation and additional feed were divided into given or not given categories. The mineral blocks were the commercially available blocks containing magnesium, iron, cobalt, copper, iodine, manganese, zinc, selenium, and sodium.

The mineral blocks were available *ad libitum* to the goats in the farms. Additional feeds given comprised of tapioca leaves, chopped and unchopped oil palm frond (OPF) and silage made from cultivated signal grass (*Bracharia humidicola*). Meanwhile, the availability and

TABLE 2
The relationship between management practices and FEC

Management practices	*n* (goats)	Mean FEC (epg)	Standard error
Grazing period			
Morning	72	3441.67[b]	528.41
Afternoon	148	721.62[a]	99.44
Mineral block supplementation			
Given	182	1388.46[a]	225.33
Not given	38	2681.58[b]	442.86
Drug type			
Ivermectin	117	1689.74	334.16
Drug combination (Benzimidazoles and Ivermectin)	103	1523.30	214.59
Breed			
Jamnapari	108	756.48[a]	126.08
Mixed breed	112	2436.61[b]	365.63
Goat source			
Local	148	2026.35[b]	292.40
Indonesia	72	759.72[a]	111.10
Type of grass			
Native grass	83	624.10[a]	112.44
Improved grass	137	2210.22[b]	309.50
Additional feed			
Given	148	721.62[a]	99.44
Not given	72	3441.67[b]	528.41
Drenching personnel			
Farm owner	112	2436.61[b]	365.63
DVS staff	108	756.48[a]	126.08

[a,b] Means within each management practice with different superscripts differ at $p < 0.05$

TABLE 3
Spearman correlation coefficient among the management practices associated with FEC

Variable	Grazing time	Mineral block supplementation	Breed	Goat source	Type of grass	Additional feed	Drenching personnel
Grazing Time	1	-0.62**	-0.70**	0.49**	-0.54**	-1	0.70**
Mineral block supplementation	-0.62**	1	0.43**	-0.30**	0.33**	0.62**	-0.43**
Breed	-0.70**	0.43**	1	0.04	0.05	0.70**	-1
Goat source	0.49**	-0.30**	0.04	1	-0.27**	-0.49**	-0.04
Type of grass	-0.54**	0.33**	0.05	-0.27**	1	0.54**	-0.05
Additional feed	-1	0.62**	0.70**	-0.49**	0.54**	1	-0.70**
Drenching personnel	0.70**	-0.43**	-1	-0.04	-0.05	-0.70**	1

** Correlation is significant at $p < 0.01$ (2-tailed)

frequency of giving the additional feed differed between the farms. The types of drug used were ivermectin and mixed combination of drugs mostly ivermectin rotated with benzimidazole. The frequency of drenching was also different for each farm. The breeds of goats raised in the six farms were mainly Jamnapari and the mixed breeds of Jamnapari crosses, Boer and Katjang. The animals were sourced locally within Malaysia and also outside of Malaysia, mainly Indonesia. As for grass, there were two types - improved grasses which are mainly creeping signal grass (*Bracharia humidicola*) as well as Napier grass (*Pennisitum purpureum*) and native grass comprising of common cow grass (*Axonopus compressus*) and buffalo grass (*Paspalum conjugatum*). The farmers or the Department of Veterinary Services (DVS) staff drenched the goats.

The systematic approaches were used for data analysis using SPSS version 17 because most of the putative practices were measured at the farm level. Therefore, it was suspected that there might be a high correlation between the management practices. To discern the effect of these practices on the FEC, a systematic approach was developed. Firstly, the association between each practice and faecal egg count was evaluated using the student t-test. Secondly, the correlation between the practices significantly associated with FEC was investigated in the first step using the Spearman's correlation coefficient to examine their relationship. Management practices with the correlation coefficient value

TABLE 4
Analysis of variance of the selected management practices

Source of variation	Df	Mean square	Sig.
Model	6	1.63	0.00*
Grazing Time	1	5.85	0.01*
Breed	1	201455.39	0.87
Goat Source	1	391841.49	0.82
Grass Type	1	413219.19	0.81
Supplement	1	4.65	0.01*
Error	214	7447687.23	
Total	220		

* Significant at $p < 0.01$

above 0.8 were considered as highly correlated and only one of the two practices was selected for further analysis based on its biological significance. Finally, the analysis of variance was performed to assess the significance of each of the practice, while simultaneously controlling for the effect of the other practices. Significance of association was considered at alpha equivalent to 0.05.

RESULTS AND DISCUSSION

Table 1 shows the similarities and differences in the management practices adopted by the six farms and their mean FEC. Table 2 presents the association between each management practice and the mean faecal egg count of animals as analyzed using the t-test. Most of the practices were significantly different except for the drug type used.

As for the drug type used by the farmers, there was no difference between ivermectin and combined drug mixture. This was due to the resistance already present in ivermectin and benzimidazoles. The high degree of resistance towards ivermectin and benzimidazoles was present in all the farms with the predomination of *Haemonchus contortus* in the post-treatment faecal cultures. The rotation of drugs could have made the resistance lower but persistent resistance or prolonged use of the drug could have rendered the inefficacy of these drug combinations compared to the usage of ivermectin alone.

As for the second analysis (Spearman correlation coefficient), all the practices were selected except for the drug type. Table 3 shows the results of the Spearman correlation. As grazing time and additional feed were correlated, grazing time was selected over additional feed. This was because grazing time is a more reliable measurement where the distinction between morning grazing and afternoon grazing is clear cut compared to the additional feed which was categorized into given and not given. Additional feed constituted oil palm frond, tapioca leaves (or silage) which are more subjective.

As breed and drenching personnel were correlated, the breed was selected instead of drenching personnel because the breed factor was clearly divided into Jamnapari or mixed breeds (Jamnapari, Boer, Katjang). Breed is also a more important biological factor compared to drenching personnel.

For the next step (i.e. the analysis of variance) only grazing time, mineral block supplementation, breed, goat source, and grass type were selected. Table 4 shows that only grazing time and mineral block supplementation were among the management practices studied that had significantly affected the worm burden in goats. The morning grazing produced a mean FEC of 3441 epg, followed by the afternoon grazing with a mean of 721 epg, and the supplementation with mineral block yielded a mean FEC of 1388 epg compared to the unsupplemented goats with a mean of 2681 epg ($P<0.05$).

The first analysis using the t-test revealed that all the management practices (except for drug type) were associated with FEC. However, in ANOVA which was used to assess the association of each practice with FEC while controlling for the other practices, only grazing time and mineral block supplementation were shown to have been associated with FEC. The data gathered in the present study showed that these two practices were mostly associated with FEC. The other practices which were significantly associated with FEC in the t-test analysis were proxy for grazing time and mineral block supplementation. Farm 1, for example, adopted afternoon grazing and had Jamnapari breed while Farm 2 used morning grazing and had the same breed (Table 1). From Table 2, morning grazing and mixed breed had significantly higher FEC compared to afternoon grazing and having Jamnapari goats. As obtained from ANOVA, breed did not really affect the FEC. Farm 2 had higher FEC compared to Farm 1 due to the morning grazing practice and not the breed factor as the Jamnapari breed did not decrease the FEC of Farm 2.

The morning grazing practice had significantly increased the worm burden in goats compared to the afternoon grazing. This was due to the presence of infective larvae on the dew-laden pasture in the mornings. Infective larvae are sensitive to weather conditions in the morning, as humidity and low degree of sunshine allow the larvae to become abundant on pasture compared to during the evening when the absence of dew prevents the larvae from being present on herbage. The findings of the present study are similar to that of Mirza and Gatenby (1993a, 1993b), whereby stall-fed control lambs maintained a geometric mean of 0.5 eggs per gram (epg). The lambs grazing in the morning, midday and afternoon had geometric mean epg values of 48, 15 and 31, respectively. The lower worm burden of the midday group was attributed to the dryness of the pasture. Kusumamihardja (1982) studied the effect of season and the time of day on the presence of nematode larvae on grass. The numbers of larvae were higher in the wet season than in the dry season, whereas, the number of larvae on the leaf blades was found to be highest in the morning. Kusumamihardja (1988) reported that in the dry season, worm burden was significantly higher in the group which was grazing in the morning than in the group which was grazing in the afternoon. However, there was no significant difference in the worm burden between morning and afternoon grazing during the wet season.

When mineral block was used as supplement, the worm burden was reduced compared to unsupplemented goats. Sani *et al*. (1995) reported there was little difference between egg counts of the animals receiving medicated urea molasses block (MUMB) and those getting unmedicated blocks. By supplementation with mineral block, the animals were able to minimize the incidence of new infections. It is assumed that provided the larval challenge is 'light', the improved nutrition provided by the blocks, irrespective of incorporation of anthelmintics, is sufficient to effectively reduce the establishment of new infections.

Further work by Maria *et al*. (1996), where urea molasses blocks medicated or not were shown to be effective in reducing new infections which lent support to this assumption. Hence, it is recommended that unmedicated mineral blocks be given when supplementation is needed, so as to reduce the likelihood of anthelmintic resistance.

Feed blocks are very popular but their high cost is a constraint in the adoption of this technology. Their popularity stems from improved productivity of the animals from increased nutrition, rather than the medication in the block. This has been clearly demonstrated by comparing the performance of goats given non-medicated and medicated blocks (Sani *et al*., 2004). Furthermore, medicated blocks are more expensive than unmedicated block and its continual long-term use is not desirable due to the concerns over the development of resistant strains of parasites (Knox, 1996).

CONCLUSION

Management practices are an important option to improve the resilience and resistance of goats in controlling parasites. The management practices selected in the study revealed their beneficial effects on worm burden in goats. Giving the goats mineral block supplementation to improve energy and protein intake and to allow the goats to graze only in the afternoon appeared to be effective in reducing FEC which extrapolates to worm burden.

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The Prevalence of Antimicrobial Resistant *Salmonella* spp. and the Risk Factors Associated with Their Occurrence in Finisher Pigs in Seberang Perai, Malaysia

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ABSTRACT

A cross sectional study to determine the occurrence and antimicrobial resistance pattern of *Salmonella* spp. in finishing pigs was carried out at 12 selected pig farms. The farm characteristics and certain management practices associated with the occurrence of *Salmonella* spp. were also evaluated. Rectal swabs were collected aseptically from 210 randomly selected finishing pigs. Suspect *Salmonella* colonies isolated were identified using a set of conventional biochemical tests and these isolates were sent to the Veterinary Research Institute (VRI) for serotyping. The antimicrobial sensitivity test was conducted on the isolates against a panel of selected antimicrobials that are commonly used in local pig production using disc diffusion method. In addition, a questionnaire on the management of farms, herd health programme, and common antimicrobial usage was collected from farm representative, resulting in variables that could be analyzed to identify factors associated with the occurrence of *Salmonella.* The results showed that 32 out of 210 finishing pigs (15.2%) tested were positive for *Salmonella typhimurium* and the isolates were detected from 9 out of 12 farms sampled (75.0%). Most of the isolates showed a relatively high level of antimicrobial resistance. The occurrence of *Salmonella* in pigs were significantly associated with (1) farms which were not equipped with footbath or vehicle wheels dip; (2) farms with vermins/birds in the surrounding; (3) farms with less frequent pen cleaning and (4) farms which are located adjacent to the neighbouring farms.

Keywords: *Salmonella*, finishing pigs, prevalence, antimicrobial resistance pattern, risk factors

INTRODUCTION

Members of the genus *Salmonella* are known for their capability to infect a broad range of host, which is considered to be a major feature in their success as food pathogens. Taylor & McCoy (1969) reported that *Salmonella* have been isolated from virtually all vertebrates from which they have been sought, with the possible exception of fish in uncontaminated waters.

Currently, more attention has been directed to *Salmonella* infection, specifically in pork and pig productions for two reasons. Firstly, in the 1990's, there was an increase in the surveillance of food-borne pathogens, giving the impression that the levels of *Salmonella* in meat products

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had considerably increased. This coincided with more attention to the emergence of virulent strains and to antibiotic resistance *Salmonella*. Secondly, from the swine medicine point of view, there have been more reports of the outbreaks of clinical *Salmonella* infection in healthy finishing pigs reared even in well-run, sanitary barns (Paul, 2002). It is important to note that preventing and limiting *Salmonella* infection in the animals at farm-level are essential, as the detection of *Salmonella* infected animals is made difficult by the absence of clinical signs. Therefore, the production of *Salmonella*-free finisher pigs is necessary to ensure safe and consumable pork and pork products (Crump *et al*., 2002).

Antibiotics kill most, if not all, of the susceptible bacteria causing an infection but leave behind the bacteria that have developed resistance. In fact, antibiotics have widely been prescribed to treat bacterial infections in humans, while many antibiotics that are commonly used in humans have also been used in animals for therapeutic and other purposes, including growth enhancement. Resistance to penicillin, a broad spectrum, started to emerge soon after its extensive introduction (Matthew *et al*., 2007). Since then, resistance to other antibiotics has also emerged.

It is a necessity to conduct epidemiological studies to determine the prevalence and possible risk factors so that *Salmonella* can be monitored and controlled at all levels of pig production. Therefore, this study was carried out to determine the occurrence of *Salmonella* in the finishing pigs, and the level of antibiotic resistance of the isolated *Salmonella* spp., as well as to identify certain management practices associated with the occurrence of *Salmonella*.

MATERIALS AND METHODS

Study Design

A small scale cross sectional study was conducted to address the objectives of the study. A total of 12 pig farms from six different pig farming areas in Seberang Perai, Pulau Pinang, were randomly selected and visited with the help of an enforcement officer from MPSP (Majlis Perbandaran Seberang Perai). The number of farms and the total of animals chosen were based on practical and financial reasons. All the farms are located at the pig farming area in Seberang Perai and the farm owners had agreed to collaborate in the present study (100% response rate).

Samples Collection and Transportation

Rectal swabs from the finisher pigs were collected aseptically using gloved hands. A total of 210 finisher pigs were randomly sampled, and about 15 to 20 rectal swabs were obtained from the finisher pigs in each farm. Each sample was inoculated immediately into a bottle containing 2 ml buffered peptone water (BPW), and kept in an ice chest containing ice-packs to maintain the survivability of the bacteria in the swab (Nielsen *et al*., 1997).

Herd Data

During the farm visits, information such as: (1) total standing pig population (SPP); (2) total and age of finisher pigs; (3) source of pigs and the management practices like: (1) total workers; (2) frequency of cleaning; (3) herd health programme, and (4) common antimicrobial usage were collected using a questionnaire. A number of closed-questions with pre-fixed answers were developed and a face-to-face interview was conducted after the samples had been collected from each farm.

Bacteriological Analysis

Each sample was pre-enriched in 2ml of BPW (Oxoid Ltd., UK) and incubated at 37°C for 18 to 24 hours. From the pre-enrichment broth, 1 ml of the media was transferred into 10 ml of Rappaport-Vassiliadis (Oxoid Ltd., UK) medium (1:10 dilution) and further incubated for 18 to 24 hours at 37°C. All the samples were then plated onto xylose lysine desoxycholate (XLD, Merck) agar and xylose lysine tergitol 4 (XLT_4,

Merck). Presumptive *Salmonella* colonies were characterised using a set of biochemical tests (Table 1) and were further confirmed by slide agglutination test using Polyvalent 'O' and 'H' antisera (Difco). The confirmed colonies were sub-cultured onto a nutrient agar slant by streaking onto its surface. The cultures were sent to VRI for serotyping (Maria *et al*., 2002).

Antimicrobial Sensitivity Test

The antimicrobial susceptibility test of *Salmonella* isolates was conducted against a panel of 25 commonly selected antimicrobials using Kirby-Bauer disc diffusion method: amikacin (30µg), amoxicillin (25µg), amoxicillin/clavulanate (30µg), ampicillin (10µg), apramycin (15µg), carbenicillin (100µg), cephalothin (30µg), chloramphenicol (30µg), ciprofloxacin (5µg), clindamycin (2µg), cephradine (30µg), cephalexin (30µg), collistin sulphate (10µg), enrofloxacin (5µg), fosfomycin (50µg), gentamycin (10µg), kanamycin (30µg), neomycin (30µg), norfloxacin (10µg), pefloxacin (5µg), tetracycline (30µg), penicillin (10µg), sulphamethaxazole (25µg), oxytetracycline 30µg, and chlortetracycline 30µg. The susceptible and resistance breakpoint levels of the antimicrobials were based mainly on those specified by the Clinical and Laboratory Standards Institute (CLSI, 2006). The inhibition zones of each antibiotic were measured after 24 hours of incubation at 37°C.

Statistical Analysis

All data were stored in SPSS v15.0 (STATCON, Witzenhause, Germany). Meanwhile, data quality was evaluated and obvious typing errors were checked against the original records and corrected. The statistical analyses involved screening of all single explanatory categorical variables by χ^2 or Fisher Exact test. Strength of association between *Salmonella* occurrence and significant risk factors were determined using the odds ratio (OR) with a 95% confidence interval. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Isolation of Salmonella *spp.*

Among a total of 210 finisher pigs, 32 pigs were found to be positive for *Salmonella* spp. The overall prevalence of *Salmonella* spp. isolated from the finisher pigs was 15.2%. The organism was isolated from nine out of twelve (75%) farms. Serotyping of the isolates in Veterinary Research Institute revealed that all the isolates were identified as *Salmonella* typhimurium.

Antimicrobial Resistance Pattern

The resistance pattern of *Salmonella typhimurium* isolated from the finisher pigs is presented in Table 2. A total of 25 antimicrobials were used to test against the *Salmonella* spp. isolated from this study. All the isolates were resistant

TABLE 1
Biochemical tests for the identification of *Salmonella* spp.

Biochemical test	Results
Triple Sugar Iron test	O/AG H_2S gas+/-
Urease test	Negative
Phenylalanine Deaminase test	Negative
Citrate test	Positive/ Negative
Arginine	Positive/ Negative
Ornithine	Positive
Lysine	Positive
Polyvalent O	Positive
Polyvalent H	Positive/ Negative

TABLE 2
Percentage of antimicrobial sensitivity in the *Salmonella* spp. (n=32) isolated from 210 finisher pigs

Antimicrobials	Potency	Sensitive	Intermediate	Resistant
		(%)	(%)	(%)
Amikacin (AMK)	30µg	32(100.0)	-	-
Amoxycillin (AML25)	25µg	7(21.8)	18(56.4)	7(21.8)
Amoxy/Clavu (AMC30)	30µg	32(100.0)	-	-
Ampicillin (AMP10)	10µg	21(65.6)	4(12.5)	7(21.8)
Apramycin (APR)	15µg	32(100.0)	-	-
Carbenicillin (CAR100)	100µg	11(34.4)	17(53.1)	4(12.5)
Cephalothin (CF30)	30µg	7(21.8)	11(34.4)	14(43.8)
Cephalexin (CL30)	30µg	32(100.0)	-	-
Cephradine (CE30)	30µg	-	21(65.6)	11(34.4)
Chloramphenicol (C30)	30µg	-	4(12.5)	28(87.5)
ChlorTetracycline	30µg	-	4(12.5)	28(87.5)
Ciprofloxacin (CIP5)	5µg	25(78.2)	7(21.8)	-
Clindamycin (DA2)	2µg	-	-	32(100.0)
Colistin sulphate (CL)	10µg	28(87.5)	-	4(12.5)
Enrofloxacin (ENR)	5µg	21(65.6)	11(34.4)	-
Fosfomycin (FOS50)	50µg	32(100.0)	-	-
Gentamicin(CN10)	10µg	32(100.0)	-	-
Kanamycin (K)	30µg	32(100.0)	-	-
Neomycin (N30)	30µg	11(34.4)	21(65.6)	-
Norfloxacin (Nor10)	10µg	32(100.0)	-	-
OxyTetracycline	30µg	-	4(12.5)	28(87.5)
Penicillin (P10)	10µg	-	-	32(100.0)
Pefloxacin (PEF5)	5µg	-	18(56.2)	14(43.8)
Sulphamethoxazole (SOX25)	25µg	-	-	32(100.0)
Tetracycline (TE30)	30µg	-	-	32(100.0)

to at least four of the antimicrobial tested. The highest frequency of resistance was detected for towards penicillin (100%), tetracycline (100%), clindamycin (100%), and sulphamethoxazole/ trimethoprim (100%). 87.5% of the isolates tested were resistant towards chloramphenicol, oxytetracycline and chlortetracycline. Meanwhile, lower percentages of resistance were observed towards cephalothin (43.8%), pefloxacin (43.8%), amoxycillin (21.8%), ampicillin (21.8%), carbenicillin (12.5%), and collistin sulphate (12.5%). 100% of the isolates tested were shown to be completely sensitive towards amikacin, amoxycillin/ clavulanate, apramycin, fosfomycin, gentamicin, kanamycin, norfloxacin, and cephalexin (8 out of 25 antimicrobials tested). More than 90.0% of the isolates were resistant to more than five antimicrobials used in the antimicrobial sensitivity test (see Table 3). Various studies have reported similar findings, where high percentages of multiple antimicrobial resistant *Salmonella* spp. were isolated from rectal swabs in pigs (Kishima *et al*., 2008; Pan *et al.*, 2010) and in other domestic species such as chicken and ducks (Tran *et al*., 2004).

Risk Factors

The analysis of the herd data showed that there were a few possible risk factors in the farm management practices, and these

TABLE 3
Multidrug resistance observed among the *Salmonella* isolates obtained from 210 finisher pigs

Salmonella Isolates (no)	Percentage of resistant to indicated number of antimicrobials				
	1-5	6-10	11-15	>16	Total (>1)
S. Typhimurium (n = 32)	9.4	34.4	43.7	12.5	100.0

might be associated with the occurrence of *S. typhimurium* (n = 32) isolated from the sampled farms (Table 4). A significant higher number of *S. typhimurium* was isolated from the farms which were not equipped with vehicle dip (χ^2= 6.65, p<0.05, OR=2.7) or footbath (χ^2= 8.263, p<0.05, OR=3.0) compared to the farms having vehicle dip or footbath. A significant number of *Salmonella* were isolated from the pigs sampled from the farms in which vermin or birds were observed in the vicinity (χ^2=3.901, p<0.05, OR=2.2). More *Salmonella* was isolated from the farms where cleaning of pens was performed only once daily (χ^2=5.422, p<0.05, OR=2.8) compared to the ones where cleaning was done more than once a day. Higher occurrence of *Salmonella* was associated with farms situated adjacent to other farms (χ^2= 4.778, p<0.05, OR=3.2).

In addition, the statistical analysis showed that farm characteristics such as: (1) the age of farm; (2) total standing pig population, and (3) total finishing pigs, as well as management practices like: (1) total number of workers in the farm; (2) age when pig is marketed; (3) source of feed; (4) source of water, and (5) frequency of feeding in a day were not associated (p>0.05) with the occurrence of *Salmonella* spp.

DISCUSSION

Salmonella typhimurium was isolated from 15.2% of the finisher pigs sampled in this study. However, the finding is inconsistent with the

TABLE 4
Significant risk factors associated with the occurrence of *Salmonella* spp.

Significant risk factors	X^2	OR[a]	95% CI[b]	p value
Availability of footbaths				
Yes		1[c]		
No	8.2663	3.0	1.44-7.15	0.004
Availability of vehicle dips				
Yes		1[c]		
No	6.65	2.7	2.34-8.30	0.010
Presence of vermin/birds				
Yes	3.901	2.2	2.03-6.75	0.048
No		1[c]		
Cleaning of pens				
Once or less	5.422	2.8	1.15-6.78	0.020
More than once		1[c]		
Location of the farm				
Adjacent to other farms/residency	4.778	3.2	1.08-9.59	0.029
Distant from other farms/residency		1[c]		

[a] OR= Odds ratio
[b] 95%CI= 95% Confidence interval
[c] Reference category

result by a study by Wondwossen *et al.* (2006), where a lower percentage of faecal *Salmonella* (4.2%) was found in conventional pig farms. This could be due to the geographical variation and the differences in the production system. *Salmonella typhimurium* have been associated with multidrug resistance, which was a major reason in its success as pathogen (Schwartz *et al.*, 1999). Therefore, their potential for survivability is much higher compared to other serotypes, specifically in adult pigs.

A high percentage of the isolates tested in this study were found to be resistant towards penicillin, tetracyline, oxytetracycline, chlortetracycline, chloramphenicol, and sulphamethoxazole. The finding is in agreement with that by Wondwossen *et al.* (2006) who found high resistance in antimicrobials such as penicillin, tetracycline, and sulpha drugs. This was most probably due to improper usage of a particular antimicrobial causing resistance to occur. A lower resistance was exhibited by *Salmonella* isolates towards carbenicillin, colistin sulphate, ampicillin, amoxycillin, cephradine, and flumequine. This could be due to the lower frequency of usage of the newly established antimicrobials in the farms which might have further reduced the risk of antimicrobial resistance. All the isolates tested were shown to be sensitive towards 8 out of 25 (amikacin, amoxicillin/clavulanate, apramycin, cephalexin, fosfomycin, gentamycin, kanamycin, and norfloxacin) antimicrobials tested. This might be due to the less usage of these antimicrobials, which in turn, did not cause any emergence of resistant *Salmonella*.

In the present study, all *S. typhimurium* isolated were resistant to at least four antimicrobials, suggesting that the multidrug-resistant (MDR) strains of *Salmonella* are prevalent. MDR can be defined as a condition where bacteria or any disease causing organism gain resistance towards the clinical doses of classical antibiotics which were previously effective against them (Cowen, 2008). The MDR strains of *Salmonella* are now frequently encountered and the rates of multidrug-resistance have considerably increased in the recent years (Paul, 2002). Some variants of *Salmonella* (e.g. phage type of *S. typhimurium*) have developed multidrug-resistance as an essential part of the genetic material of the organism, and are therefore likely to preserve their drug-resistant genes and enhance their survivability (Rowe *et al*., 1997).

The current study found that higher *Salmonella* prevalence was associated with farms, which were not equipped with vehicle dip or footbaths. During transportation of pigs and feed (Berends *et al.*, 1996), *Salmonella*-negative finishing pigs might be infected from the previously contaminated trucks that had not been thoroughly cleaned (Fedorka-Cray *et al.*, 1997). Meanwhile, the presence of vermins and birds could be a source of *Salmonella* transmission, shedding and contaminating the farm and the feed with *Salmonella* (Davies & Wray, 1997; Funk *et al*., 2001). Therefore, controlling birds and vermins in the farm, as well as keeping pets, such as cats and dogs out of the vicinity, will help to prevent the introduction of *Salmonella* into the farm (Letellier *et al*., 1999).

The present study have also shown that a high number of *Salmonella* could be isolated from pigs where cleaning of the pens were done less frequently. Mannion *et al*. (2007) reported that high prevalence farms tended to have more residual contamination of feeders and equipment after barn cleaning than low prevalence farms, suggesting that more stringent and frequent cleaning could be associated with decreased prevalence. Therefore, pens should be cleaned more frequently to avoid accumulation of *Salmonella* in the environment. *Salmonella* could be transmitted through dust (Baggesan *et al.*, 1996; Edel *et al.*, 1970) and aerosols (Lever & Williams, 1996) which might have increased the risk of *Salmonella* transmission in farm located adjacent to other neighbouring farms. Furthermore, the movement of individuals or farm personnel between the farms might also increase the risk of *Salmonella* contamination.

CONCLUSIONS

The high prevalence of *Salmonella* in the local pig farms and the level of antimicrobial resistance in *Salmonella* have raised concerns and constituted a real threat to the public health. Thus, proper drug therapy practices and high hygiene standard in the farm can considerably reduce *Salmonella* contamination in the farm, and these will ultimately benefit the consumers. The findings in this study may not necessarily reflect the general contemporary production environments as the epidemiology of *Salmonella* infection in swine farm is very complex. However, these results may be used as a basis to show that pigs may pose as potential reservoir in harbouring multidrug-resistant *Salmonella*. More research is still needed to identify the possible risk factors associated with the occurrence of *Salmonella* spp. in pig farms so that intervention can be done to keep *Salmonella* levels low. This study reinforces the importance of good farm management and prudent use of antimicrobials to control the disease at all levels of animal production effectively.

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Short Communications

Microsatellite Markers for the Identification of Commercially Important Groupers *Epinephelus lanceolatus*, *Cromileptes altivelis* and *Epinephelus fuscoguttatus*

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ABSTRACT

The Giant Grouper (*Epinephelus lanceolatus*), Mouse Grouper (*Cromileptes altivelis*) and the Brown Marbled Grouper (*Epinephelus fuscoguttatus*) are all found in the coastal waters of Sabah, Malaysia; they are listed as vulnerable, threatened and near-threatened, respectively, according to the IUCN Red Data list. Based on microsatellite loci, molecular markers can be applied as versatile tools for forensic detection, population genetic studies and the development of genomic databases which can subsequently be incorporated into viable conservation programmes. This paper describes the development of molecular markers for the three species of grouper. DNA was extracted from adult specimens of the three species, which are currently maintained at the Borneo Marine Research Institute Aquaculture facility, subject to DNA amplification using a multiplex PCR enrichment procedure, and the PCR products were ligated onto a pJET 1.2 blunt cloning vector. This was followed by sequencing and the development of 24 specific molecular markers for each of the three species. These markers have a potential application for the identification and forensic detection of these species in transit and can be adopted within the context of a strategy for the conservation and management of Malaysian fisheries resources.

Keywords: *Epinephelus fuscoguttatus*, *Epinephelus lancelolatus*, *Cromileptes altivelis,* molecular markers

INTRODUCTION

Groupers belong to the subfamily *Epinephelinae* of the family *Serranidae* and inhabit reefs from the tropics to the sub-tropical waters. Many of the species have been classified as vulnerable as in the case of the Giant groupers (*E. lanceolatus*), according to the IUCN Red Data list. The high demand for reef fishes in the developing nations of Southeast Asia has further exacerbated the decline of wild Grouper populations and led to the necessity for interventions such as the protection of spawning sites (Cesar *et al.*, 2003). Meanwhile, the development of molecular markers for the forensic detection of these endangered and economically important species forms the basis of this investigation, which is directed towards developing specific DNA based molecular markers for the forensic identification of *E. lanceolatus*, *E. fuscoguttatus*, and *C. altivelis*.

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Several previous studies done on *E. lanceolatus* (Zeng *et al*., 2008) have focused on eight polymorphic microsatellite loci; however, for the purpose of forensic identification, emphasis has been placed on the development of molecular markers, based on the relatively stable non-hyper-variable genomic regions.

MATERIALS AND METHODS

The adult specimens of *C. altivelis*, *E. fuscoguttatus* and *E. lanceolatus* were selected for the sample collection from the fish hatchery at Borneo Marine Research Institute, Universiti Malaysia Sabah. Fin clips of approximately 1 cm^2 were excised. Meanwhile, total DNA was extracted using the method described by Doyle and Doyle (1987), with a minor modification of the extraction buffer, to which 500 µg/ml final concentration of Proteinase K (Sigma) was added. Amplicons containing genomic repeat motifs generated using three Inosine containing degenerate primers, with a range of repeat motifs, BRICTT10 [5'-GG$(CTT)_{10}$III-3'], BRICAA8 [5'-GG$(CAA)_8$III-3'] and BRIAGG8 [5'-GG$(AGG)_8$ III– 3'] (where I = Inosine), were used in PCR amplification to isolate the range of repeat motifs. PCR amplification was carried out in a total volume of 20 µL containing 50 ng of template DNA, 1.5 mM $MgCl_2$,1X PCR buffer containing 10 mM Tris-HCl (pH = 8.0), 50 mM KCl, 1 U Taq DNA polymerase (Qiagen), 20 pmol of primer (BRICTT10, BRICAA8 and BRIAGG8), 0.2 mM of dATP, dGTP, dCTP and dTTP. Amplification was carried out in a thermocycler (MJ Research thermal cycler) with an initial denaturation at 94°C for 3 minutes, and this was followed by 30 cycles of 30 s at 94°C, 40 s at 55°C or 58°C, 1 minute at 72°C and a final extension step of 5 min at 72°C. The amplification products were separated by electrophoresis on a 2% TBE Agarose gel with a 1 Kb DNA ladder (Promega) as a size standard. PCR products containing distinct amplicons, with a size in the excess of 200 bp, were purified using a PCR purification kit (Qiagen), while 2 µL of the purified products were cloned onto a pJET1.2 blunt cloning vector and transformed into chemically competent TOP10F E. coli cells, according to the instructions from the manufacturer (Fermentas). A total of 20 recombinant clones from each species were selected randomly from Luria-Bertani plates containing Ampicillin (100 mg/ litre) and X-Gal (50 mg/ litre). Plasmids were extracted and purified using GeneJET plasmid purification kit (Fermentas). Plasmids with an insert size in excess of 300 bp were sequenced using BigDye Terminator 2.0 Cycle Sequencing Ready Reaction Kit (Applied Biosystems) on an ABI Prism 377 DNA sequencer. The characterization of specific genomic loci was done using specific primer pairs designed to flank the regions containing microsatellite repeat motifs using the online software PRIMER 3 (Rozen & Skaletsky, 2000). A total of 24 primer pairs were designed, synthesized, and tested across five representative specimens from each of the three species. The PCR amplification was carried out in a total volume of 20 µL containing 50 ng of template DNA, 1.5 mM $MgCl_2$,1X PCR buffer containing 10 mM Tris-HCl (pH = 8.0), 50 mM KCl, 0.25 U Proofreading Taq DNA polymerase (Qiagen), 10 pmol of each forward and reverse primer, 0.2 mM of dATP, dGTP, dCTP, and dTTP. The amplification was carried out in a thermocycler (MJ Research thermal cycler) with an initial denaturation at 94°C for 3 minutes, followed by 30 cycles of 30 s at 94°C, 40 s at the specific annealing temperature, 40 s at 72°C and a final extension step of 10 min at 72°C. The amplification products were separated by electrophoresis on a 2% TBE agarose gel (Promega), with a 100 bp DNA ladder (Promega) as a size standard. The gel was stained with Ethidium Bromide (5 µg/ ml) and the bands were scored using the gel documentation system ALPHAIMAGER 2000.

RESULTS AND DISCUSSION

The amplification of the DNA with the primers developed resulted in distinct amplification profiles for each of the three species of groupers (*Fig. 1*). The specificity of the amplification was improved by increasing the number of the

repeat motifs as in the case of primer BRICTT10 (*Fig. 2*); this is an indicative of the rarity of the repeat motifs within the genome in direct relation to the repeat frequency. Meanwhile, the application of degenerate primers containing inosine increased the probability of PCR amplification of microsatellites, and this can be attributed to the fact that microsatellite repeat units are likely to contain interruptions in the form of nucleotide additions and substitutions

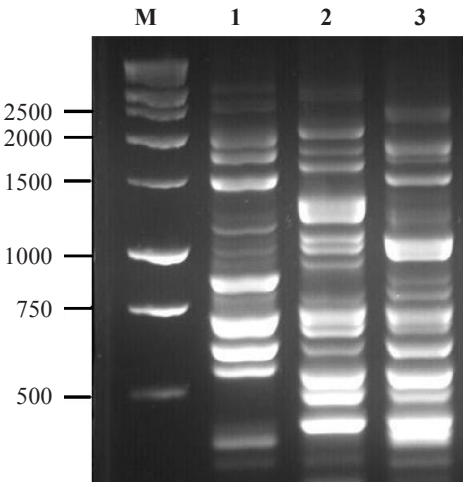

Fig. 1: 2% TBE agarose gel electrophoresis analysis of PCR amplicons generated as a result of the amplification of grouper DNA with multiplex primer BRICAA8, according to the conditions described in the text. Lane M: 1 Kb DNA ladder, Lane 1: C. altivelis*, Lane 2:* E. fuscoguttatus*, Lane 3:* E. lanceolatus *(all the marker sizes are indicated in base pairs)*

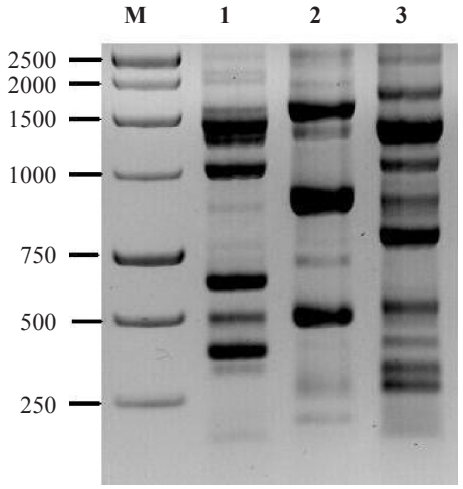

Fig. 2: 2% TBE agarose gel electrophoresis analysis of PCR amplicons generated as a result of the amplification of grouper DNA with multiplex primer BRICTT10, according to the conditions described in the text. Lane M: 1 Kb DNA ladder, Lane 1: C. altivelis*, Lane 2:* E. fuscoguttatus*, Lane 3:* E. lanceolatus

TABLE 1
Microsatellite markers developed for the forensic identification of *Epinephelus lanceolatus* (EL), *Epinephelus fuscoguttatus* (EF) and *Cromileptes altivelis* (CA) indicating specific locus, primer sequence (5' – 3'), observed size in base pairs (bp) and annealing temperature (°C)

No.	Locus	Primer sequence 5' – 3'	Observed size (bp)	Annealing temperature (°C)	GenBank accession number
1.	CAA01B	F: GAGGAGTTCAAAGCCTCCAA R: CCCTTTTCCCAAAATGACAA	311	58	GQ912309
2.	CAA02B	F: TAACTTGCCAAGCATCAGCA R: GCACTTTCCTCCGAAAAACA	312	60	GQ912310
3.	CAA03B	F: TGCTTGACCAAAACACAATGA R: GCTAGGCGGAAGTGACAAAG	371	60	GQ912311
4.	CAA04B	F: GCGGTTAAACAGAGGGGATT R: ACTCGTGTTCCCTCCTGCT	164	58	GQ912312
5.	CAA05B	F: TTTAACCCGGTCAACTCCAG R: GACGCAGTTTCAATGCAAAA	535	58	GQ912313
6.	CAA06B	F: ATCAGTGTCCTGCCACCTTC R: TACTCCACATGCTGGCTGTC	340	59	GQ912314
7.	CAB01A	F: GACTGAATTTGGGGACCAAG R: CAATGTCGACGTCGCTAAAC	222	58	GQ912315
8.	CAB06B	F: TTCCTTCAGCAACAAACACG R: CCGCAAACAGTGCTAAACAA	315	58	GQ912318
9.	EL09A	F: TGCTGGTTTTTACGGAGACC R: TGTCTGCACCACCTGTCATT	157	58	GQ912329
10.	EL10A	F: GCTCAGCTGTTGAAAACACG R: GCTCCTCCGAAATGTCTCTG	308	58	GQ912330
11.	EL16A	F: TTCTTCCTCTGCTGTCTTTTCC R: TTACGTTTCCAGAGCACCAA	301	58	GQ912331
12.	EL19A	F: GAATCTCCTGCACCTCTTGC R: TGCTGGAGCTGTATCCTCCT	397	58	GQ912332
13.	EL20A	F: CGACAAAACCGGGATTAAAA R: GGAAAGGGAAGTTGGGAGAG	304	60	GQ912333
14.	EL21A	F: ACTTCCCTCCAATGCTTCAA R: CCTTCGTCCACCATCAGTCT	302	58	GQ912334
15.	EL22A	F: GCACAAGCCTAGCCCTACTG R: TTGGGTCCAATGGAACATTT	399	58	GQ912335
16.	EL13A	F: AATGAGCACCTGGAGACCAC R: TTTTCAGCCTTCCTCTCCTG	352	59	GQ912336
17.	EFX2B	F: GCGCTGCTGTACAACAAGAA R: TCAGCAGGTGAACTGAGGTG	420	58	GQ912319
18.	EFX7A	F: AGCACGGTCTGTGTGTCTTG R: TGCCACAAAACTAAGAAAGGAA	185	58	GQ912320
19.	EFX8A	F: CGTCACTGACTGCCAAGAAA R: GAGCCAGGACCAGTTGTAGC	187	58	GQ912321

Table 1 (Continued)

20.	EFX10B	F: GTTGTTGTTGTTGCCAATCG R: GAGTCAGTGAGCGAGGAAGC	346	59	GQ912322
21.	EFX11B	F: TTGGCAGGTGTCTCTCTCCT R: ACAGCGGTGGAAGGTTACTG	211	58	GQ912323
22.	EFY1B	F: GAAGAGAACCAGTGGGGACA R: AGAGGTCGCCATCTGACATC	484	58	GQ912325
23.	EFY7A	F: CACAGGCTGTCAAACAGGAA R: TCGGGAGAATGTGAAGCTCT	228	58	GQ912326
24.	EFY8A	F: ACTGCTGCGTCAAGAAGACA R: ATCAGTGTCCTGCCACCTTC	216	58	GQ912327

resulting from strand slippage during replication (Schlötterer & Tautz, 1992) and the exclusion of degenerate bases which reduces the likelihood of amplification of loci containing imperfect microsatellite repeats. A similar approach was undertaken by Yaish & Pérez de la Vega (2003) for the isolation of microsatellite loci from *Phaseolus vulgaris*.

Meanwhile, the sequencing of each of the amplicons yielded distinct sequences for each of the species. A search for similarity using the NCBI blastn algorithm indicated that each of the sequences was novel and had not been previously described. Specific primer pairs were designed for each of the sequences (Table 1) and were determined to be species specific for each of the three species on which they were tested. These markers can be used for multiplex fingerprinting of the three grouper species, particularly for monitoring fisheries stocks and in the aquaculture industry. The ongoing focus of this investigation is the development of molecular markers used to determine Mendelian inheritance patterns and genetic recombination in grouper hybrids.

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Sequence Variation in the Cellulose Synthase (*SpCesA1*) Gene from *Shorea parvifolia* ssp. *parvifolia* Mother Trees

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ABSTRACT

Cellulose synthase (CesA) is a key enzyme involved in the regulation of cellulose biosynthesis pathway. It is heritable and important in determining the variability of wood. In particular, it provides greater impact on the design of future genetic improvement strategies in the production of high quality wood. Thus, the molecular diversity of partial *SpCesA1* genomic DNAs (802 bp) generated through PCR amplification was examined in this study, and this followed by sequencing from five selected *Shorea parvifolia* ssp. *parvifolia* mother trees. The consensus sequences were aligned to detect the presence of single nucleotide polymorphisms (SNPs). In total, seven SNPs were detected at nucleotide 58, 66, 69, 194, 224, 376 and 448. Interestingly, one single base pair InDel polymorphism was also detected at nucleotide 67. On average, one SNP at every 109 bp of the sequence data was detected. However, this result was obtained from a study of partial *SpCesA1* genomic DNA of 802 bp. Two possible restriction enzymes were detected on two SNP sites of partial *SpCesA1* genomic DNA. These included *Ear*I (5'-GAAGAG-3') and *Eco*RI (5'-GAATTC-3'), which were recognized and later cut at nucleotides 48 and 370, respectively. The exclusiveness of the restriction enzymes *Ear*I and *Eco*RI obtained for SNPs at nucleotides 58 and 376, respectively, could be useful for the development of cleaved-amplified polymorphic sequence (CAPS) markers which could also be used to understand the molecular diversity of the *CesA* genes in tropical tree genomes.

Keywords: Cellulose synthase, wood formation, *Shorea parvifolia* ssp. *parvifolia*, PCR, single nucleotide polymorphisms, molecular marker

INTRODUCTION

Wood consists of 40 to 50% cellulose. The basic structural units are the crystallized microfibrils (MFs) formed when multiple hydroxyl groups on the glucose residues from one chain of cellulose form hydrogen bonds with the oxygen molecules on the other chain, holding the chains firmly together side-by-side. The water-insoluble cellulose MFs are associated with the mixtures of soluble non-cellulosic polysaccharides, the hemicelluloses, which account for about 20% of the dry weight of wood (Plomion *et al.*, 2001). Xyloglucan is an example of these hemicelluloses. Xyloglucan binds non-covalently to cellulose MFs, thereby

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creating a strong cellulose-xyloglucan network that accounts for the rigid structure of plant cell walls.

Cellulose synthase (CesA) is the key enzyme involved in the regulation of the cellulose biosynthesis pathway (Campbell *et al.*, 1997). They are heritable and important in determining the variability of the wood. Hence, this presents an opportunity to select for improved wood properties such as superior product quality (Guimaraes *et al.*, 2007). Traditional chemical and technological assays of such selection are costly and the phenotype assessment is a complex process due to the long generation intervals and poor juvenile-mature trait correlation of wood species (Grattapaglia, 2004). The objective of this study was to determine the feasibility of finding single nucleotide polymorphisms (SNPs) from the cellulose synthase (*CesA*) gene in five selected *Shorea parvifolia* ssp. *parvifolia* mother trees. SNPs are the sequences in the genome of an organism that differs by a single nucleotide between the individuals of the same species (Cargill *et al*., 1999; Thumma *et al*., 2005; Guimaraes *et al*., 2007; Ramos-Onsins *et al*., 2008).

Shorea parvifolia ssp. *parvifolia*, which is locally known as meranti sarang punai, is one of the most valuable and sought after commercial timber tree species belonging to Dipterocarpaceae family. It has been identified as one of the potential fast growing indigenous species that grows well in lowland to upper hill land at the altitude of up to 700m (Newman *et al.*, 2006). The trees are important for producing plywood, veneer, furniture, hardboard, and particleboard.

MATERIALS AND METHODS

Total Genomic DNA Isolation

Fresh young leaves from five randomly selected *S. parvifolia* ssp. *parvifolia* mother trees (2a, 3a, 4a, 6a, and 12a) were collected from the Semengoh Forest Reserve, Kuching, Sarawak. Total genomic DNA was extracted using a modified CTAB method from Doyle and Doyle (1990) and purified using the Wizard® Genomic DNA Purification Kit (Promega, USA). The concentration of DNA was quantified using a spectrophotometer (Lambda 25 UV/VIS Spectrometer, Perkin Elmer, USA).

Primer Design and PCR Amplification

Primers SPPT3-F: 5'-CACACGATCGT TATGCCAAC-3' and SPPT3-R:5'-AGC TCTTTTTGGCAT GCAGT-3' were designed based on the full-length cDNA of *SpCesA1* (GenBank accession number: GQ338420) using Primer3.0 software. This primer pair produced an amplicon of size ~800 bp. The PCR reaction was carried out in a Gradient Palm-Cycler™ (Corbett Research, Australia) with 2 minutes of initial denaturation at 95ºC, followed by 35 cycles of 30 seconds of denaturation at 94ºC, 30 seconds of annealing at 61ºC, 30 seconds of elongation at 72ºC, and ending with 8 minutes of the final elongation at 72ºC. The total 25 µl PCR reaction volume contained 1x PCR buffer (200 mM Tris-HCl, 500 mM KCl, pH8.4), 1.5 mM $MgCl_2$, 0.2 mM of dNTPs, 5 pmol of primers, 1 unit *Taq* DNA polymerase (Promega, USA) and 10 ng of template DNA.

Cloning and DNA Sequencing

The PCR amplicons (~800bp) were then gel purified using QIAquick® Gel Extraction kit (QIAGEN, Germany) and cloned into pGEM®-T Easy vector (Promega, USA). Three clones for each mother tree were selected for the plasmid extraction using Wizard® *Plus* SV Minipreps DNA Purification System (Promega, USA) and sequenced using the Big Dye (dRhodamine) terminator cycle sequencing-ready reaction kit (Applied Biosystems, Foster City, CA).

Data Analysis

The raw data of DNA sequences were checked and edited using Chromas Lite 1.0 (http://www.technelysium.com.au/chromas 14x.html). The 3 *SpCesA1* sequences for each mother tree were aligned using CLC Free Workbench 4.0 (CLC Bio, Denmark) to obtain the consensus sequences

and then subjected to BLASTn similarity search against the non-redundant nucleotide database. The consensus sequences for five mother trees were then aligned using CLC Free Workbench 4.0 (CLC Bio, Denmark) and visually inspected for any base difference among the five mother trees. Meanwhile, *in silico* restriction of the consensus *SpCesA1* sequences was done using NEBcutter V2.0.

RESULTS AND DISCUSSION

For each mother tree, a total of three clones were selected, sequenced, and aligned using CLC Free Workbench 4 (CLC Bio, Denmark). Mother tree 12a did not show any nucleotide variation while the others (2a, 3a, 4a, and 6a) showed a variation among the clones from the same tree. In general, all 5 partial *SpCesA1* genomic DNAs for five selected mother trees (2a, 3a, 4a, 6a, and 12a) showed a high degree of similarity with *CesA Betula* spp. (91%), poplar (88%), and eucalypt (85%), as shown in Table 1.

The consensus sequences of 2a, 3a, 4a, 6a, and 12a were aligned together for manual detection of single nucleotide polymorphisms (SNP) (*Fig. 1*). The alignment result revealed that nucleotide variations occurred between mother trees 3a, 4a and 6a and mother trees 2a and 12a. A total of 7 SNPs were detected at nucleotide 58, 66, 69, 194, 224, 376, and 448. One most interesting observation from the alignment result was the discovery of a single base-pair Insertion-Deletion (InDel) polymorphism at nucleotide 67 for mother trees 2a and 12a. A deletion of adenine at nucleotide 67 of the partial *SpCesA1* genomic DNA of mother trees 2a and 12a was confirmed by ClustalW analysis (http://www.ebi.ac.uk/Tools/clustalw2/ index.html, Larkin et al., 2007) on the full-length SpCesA1 cDNA (Genbank accession no.: GQ338420) with *Eucalyptus grandis CesA3* genomic DNA (Genbank accession no.: EU165713), *E. grandis* full-length CesA3 cDNA (Genbank accession no.: EU165710) and the consensus sequence of all the five partial *SpCesA1* genomic DNAs. This explains the notable size differences between the amplicons generated from a single primer set.

SNP differs from InDel, whereby SNP involves the alteration of a single nucleotide at a specific location in the genome, while InDel includes an insertion or deletion of a number of nucleotides relative to the other. InDel has been related to genome size evolution where the possibility of illegitimate recombination explains genomic downsizing (Grover *et al*., 2008). Zhang *et al*. (2008) reported that many of the small InDels detected in rice hybrids resulted in the formation or disruption of putative *cis*-regulatory elements which were closely associated with the expression of transcription factors and thus, agronomic performance. This is also consistent with the finding by Plantegenet *et al*. (2009) who reported that the preponderance of InDels found in the exons of the *Arabidopsis* accessions Eil-0 and Lc-0 caused drastic effects on gene integrity, specifically on the gene representing expression level polymorphisms.

The partial *SpCesA1* genomic DNA contains coding and non-coding regions. Intron-exon boundaries were predicted from the ClustalW analysis carried out to verify the InDel previously. A schematic diagram was drawn to represent the predicted intron-exon boundaries (*Fig. 2*). The predicted partial *SpCesA1* genomic DNA intron-

TABLE 1
BLASTn output for amplified partial *SpCesA1* genomic DNA of 802 bp

Organism	GenBank accession no.	Similarity (%)	E-value
Betula luminifera	FJ410445.1	91	6e-53
Betula platyphylla	EU591531.1	91	6e-53
Populus trichocarpa	XM 002325086.1	88	3e-51
Eucalyptus grandis	DQ014507.1	85	2e-45

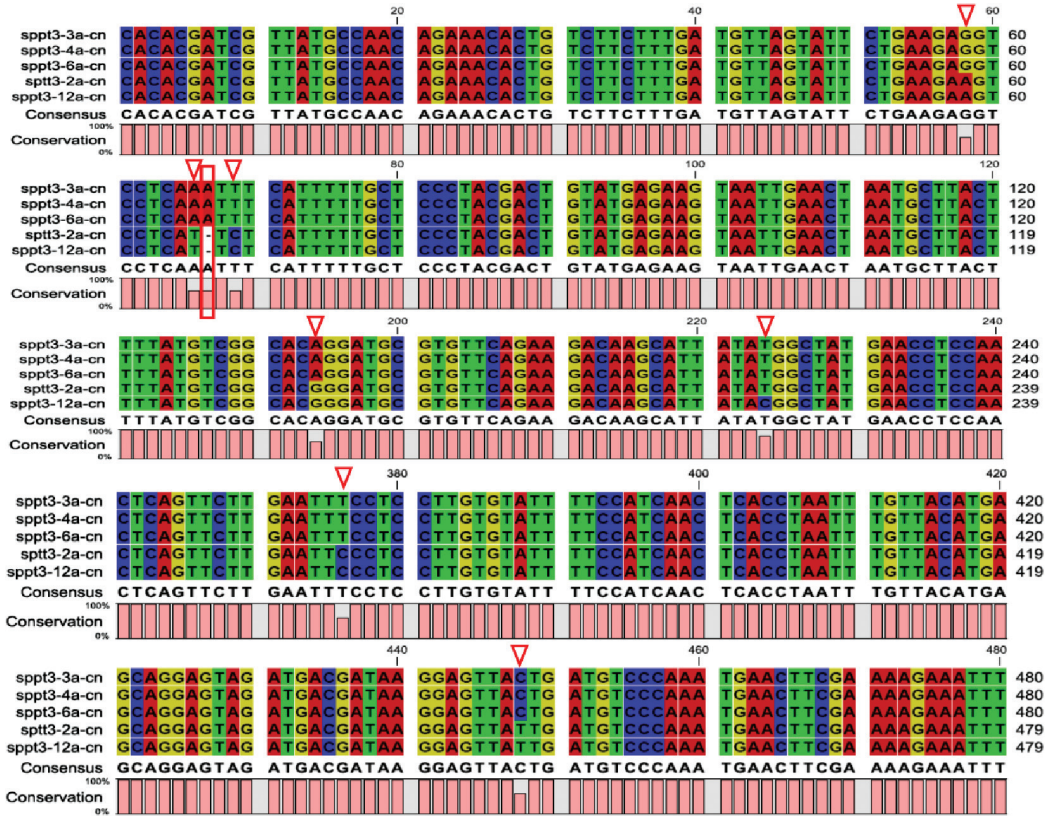

Fig. 1: Alignment of consensus sequences for 2a, 3a, 4a, 6a, and 12a using CLC Free Workbench 4 (CLC Bio, Denmark). Seven SNPs were detected at nucleotides 58, 66, 69, 194, 224, 376, and 448 (red triangle) and one INDEL detected at nucleotide 67 (red box)

exon junctions are conserved as they are also found in *E. grandis CesA3* genomic DNA. There is only one non-coding region detected from the predicted partial *SpCesA1* genomic DNA, with a size of 36 bp from nucleotide 389 to 425.

The data obtained from this study revealed that the chances of detecting SNPs in the partial *SpCesA1* genomic DNA are high in the exons, with about one SNP in every 109 bp compared to zero SNP in the intron region (Table 2). However, this statement was made based on the study of a partial *SpCesA1* genomic DNA of 802 bp. Joshi (2003) reported that even a single base pair mutation in the coding region of the *CesA* gene in *Arabidopsis* would impact the process of cellulose biosynthesis. This is consistent with the findings that the *irx*3 (irregular xylem 3) point mutation in *AtCesA7* shows a defect in the xylem secondary cell wall formation leading to the weakened walls of the treachery elements that later collapse upon themselves (Turner & Somerville, 1997; Taylor *et al.*, 1999). The Indel detected in this study was located in the coding region of the partial *SpCesA1* genomic DNA.

Two possible restriction enzymes were detected for the two SNP sites of the partial *SpCesA1* genomic DNA by using NEBcutter V2.0. They are *Ear*I (5'-GAAGAG-3') and *Eco*RI (5'-GAATTC-3') which recognize and later cut at site numbers 48 and 370, respectively. *Ear*I produces two fragments sized approximately 48 bp and 754 bp, whereas *Eco*RI produces two fragments sized approximately 370bp and 432 bp. The discovery of SNPs is of

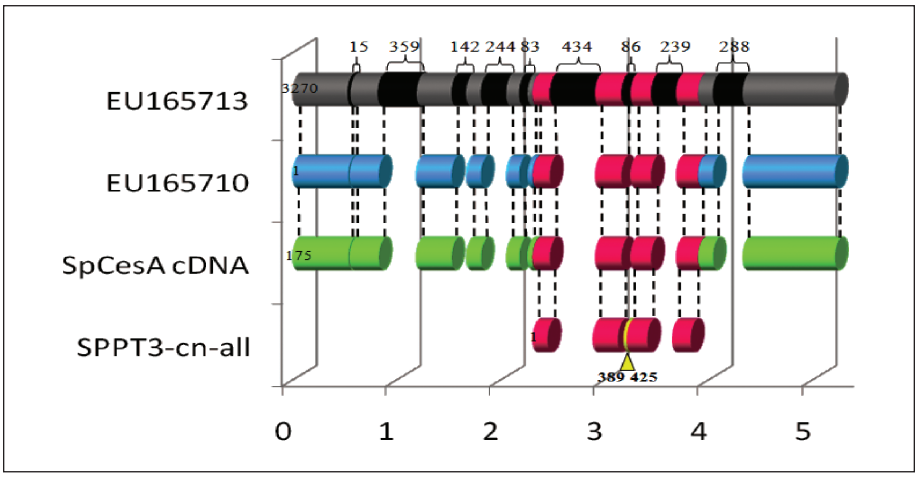

Fig. 2: Comparison of the gene structure of Eucalyptus grandis *CesA3 genomic DNA (Genbank accession no.: EU165713; 8771 nt),* E. grandis *CesA3 mRNA (Genbank accession no.: EU165710; 3331 nt), full-length SpCesA1 cDNA (Genbank accession no.: GQ338420; 3308 nt), and consensus sequence of all the five partial SpCesA1 genomic DNAs. Figures before each sequence denote the start nucleotide in the alignment. Coloured cylinders represent exons and black cylinders represent introns with the number of bases indicated above them. The intron portion as predicted from the partial SpCesA1 genomic DNA is shown in solid yellow. Dotted lines connecting different genes indicate conserved intron-exon junctions.1 unit axis: 1kb*

TABLE 2
Sequence variations within partial *SpCesA1* genomic DNAs among five selected *S. parvifolia* ssp. *parvifolia* mother trees

Region/ Parameter	CesA
Exons	
Total no. of SNPs	7
bp in fragment sequenced	766
SNPs per bp	~ 109
Introns	
Total no. of SNPs	-
bp in fragment sequenced	36
SNPs per bp	-

advantage as many SNPs alter the sites cleaved by restriction enzymes and hence, can be used as cleaved-amplified polymorphic sequence (CAPS) markers (Konieczny & Ausubel, 1993). CAPS markers are amplified by PCR; the amplified DNA will be cleaved with the carefully chosen restriction enzyme, such as that through *in silico* restriction analysis, and the cleaved products are examined on agarose gels. CAPS that posses the property of being co-dominant allows the differentiation of heterogygotes and homozygotes.

CONCLUSIONS

This study has demonstrated that the PCR amplification, followed by sequencing using primers designed from the full-length of the *SpCesA1* cDNA sequence, is an effective technique for obtaining genomic clones and classifying molecular diversity in the cellulose synthase gene (*CesA*) in *S. parvifolia* ssp. *parvifolia*. SNPs in those gene sequences that are significantly associated with the changes of cellulose content, and composition can then be used for early selection of planting material at the seedling stage. In particular, this information is essential to further understand the molecular diversity of the *CesA* genes in tropical tree genomes, as this could have many fundamental and commercial implications.

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Isolation and Identification of *Fusarium* Species Associated with Fusarium Ear Rot Disease of Corn

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ABSTRACT

Fusarium species associated with corn (*Zea mays*) are very diverse and widely distributed throughout Malaysia. Out of 657 samples cultured, a total of 220 *Fusarium* isolates were obtained from corn plants showing the typical symptoms of Fusarium ear rot in 12 locations throughout Malaysia. All the isolates were identified into 10 species based on the morphological characteristics that emphasized on growth rates, colony features and microscopic characteristics. A total of 117 *Fusarium* isolates were classified into four species in the section Liseola and their allied, tentatively identified as *F. proliferatum* (58), *F. subglutinans* (34), *F. verticillioides* (24), and *F. nygamai* (1). Meanwhile, *F. proliferatum* was the most prevalent species in all the sampling areas. 103 isolates, which were classified into six other *Fusarium* species belonging to different sections, were also isolated and identified, and these included *F. semitectum* (47), *F. oxysporum* (20), *F. pseudograminearum* (19), *F. solani* (15), *F. equiseti* (1), and *F. longipes* (1). *F. semitectum* was the highest among other common saprophytic fungi in corn. *F. pseudograminearum* was only isolated from the samples obtained from Cameron Highlands, Pahang. In term of species diversity, *Fusarium* species was the highest obtained in Semenyih, Selangor, with H`=1.72.

Keywords: *Fusarium* species, Fusarium ear rot, diversity, corn, morphology

INTRODUCTION

Genus *Fusarium* is classified under phylum Ascomycota and ubiquitous fungi that are extensively distributed worldwide, from the temperate to the tropical regions (Leslie & Summerell, 2006). *Fusarium* species are commonly reported as endophytes, saprophytes, and pathogens of various plants, especially economically important crops, including corn (Nelson *et al*., 1983; Burgess *et al*., 1994). Corn (*Zea mays*) belongs to dicotyledonous angiosperm plant and it is a member of the grass family Poaceae.

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Fusarium species can cause plant diseases, such as wilt, rot, abnormal growth and decay, on vegetables, wood, herbs, and ornamental plants. Besides being plant pathogens, *Fusarium* species may also produce secondary metabolites, such as mycotoxins (i.e. beauvericin, fumonisins, and moniliformin), as well as phytotoxins (fusaric acid and gibberallic acid) problems (Booth, 1971; Summerell *et al*., 2003). Fumonisins and moniliformin can cause serious mycotoxicoses on humans and animals (Summerell *et al*., 2003), whereas, fusaric acid and gibberellic acids can respectively lead to stunting and abnormal elongation on the growth of plants.

Meanwhile, the *Fusarium* species in the section Liseola and their allies have been reported as potential pathogens on corn and caused Fusarium ear rot disease. However, the identity and aetiology of this pathogen is still highly debated. Therefore, this study was conducted to isolate and identify the *Fusarium* from the samples showing typical symptoms of the disease. Recently, *Fusarium* species such as *F. proliferatum*, *F. oxysporum*, *F. nygamai*, *F. semitectum*, *F. solani*, *and F. verticillioides* were successfully isolated from corn showing typical symptoms of ear rot disease in four states of Malaysia, namely, Perlis, Pulau Pinang, Sabah and Sarawak (Darnetty *et al*., 2008). However, no report is available on the distribution and diversity of *Fusarium* isolates obtained from the west coast (Selangor), east coast (Pahang) and Southeast areas (Johor) of Peninsular Malaysia.

MATERIALS AND METHODS

Corn Samples

A total of 657 corn samples were obtained from 12 main corn growing areas throughout Malaysia. All the samples were surface sterilized with 10% Clorox® and rinsed in several changes of sterile water.

The Isolation of Fusarium *Isolates and Monospore Culture*

The samples were placed on semi-selective medium for *Fusarium*, peptone pentachloronitrobenzene agar (PPA) as described by Nash & Snyder (1962), and incubated for 7 days under standard growth conditions (Salleh & Sulaiman, 1984). The cultures were single-spored following a standard protocol by Burgess *et al.* (1994). After 7 days of incubation, the cultures were transferred onto potato dextrose agar (PDA) and carnation leaf agar (CLA; Fisher *et al*. 1982) for species identification.

Morphological Characteristics and Species Identification

The cultures on PDA were used for observing the macroscopic characteristics such as colony features, growth rate and pigmentation. For microscopic characterization, pure cultures were transferred onto CLA and soil extract agar (SEA; Klotz *et al*., 1988). The microscopic characteristics such as conidia ontogeny, as well as the presence of chlamydospores and types of conidiophores were examined following the procedure by Burgess *et al*. (1994) and Leslie & Summerell (2006). The observations were done using a light microscope (Olympus model BX-50F4) and photographed using a JVC camera model KY-F55BE, with an image analyzer-SIS programme. The *Fusarium* isolates were identified based on the morphological characteristics into species level following Nelson *et al*. (1983), Burgess *et al*. (1994) and the Fusarium Laboratory Manual (Leslie & Summerell, 2006). The pure cultures were maintained on water agar (WA) as short-term working cultures (Burgess *et al*., 1994).

Diversity of the Fusarium *Species*

The diversity of the *Fusarium* species isolated within Peninsular Malaysia was calculated based

on Shannon-Weiner Index (Spellerberg, 2008), as follows:

$$H' = -\sum_{i=1}^{s} p_i \ln p_i$$

where: H' = value of Shanon-Weiner Index

$\sum$ refers to "the sum of"

there are *s* species in the community

p_i = is the relative abundance (proportion) of the i species in the community

ln = natural log

RESULTS AND DISCUSSION

A total of 220 isolates of *Fusarium* were obtained from the corn plant samples showing typical symptoms of *Fusarium* ear rot disease in 12 locations throughout the main corn growing areas in Malaysia. The typical symptoms of *Fusarium* ear rots are a white to pink- or salmon-coloured mold (fungal mass), beginning anywhere on the ear or scattered throughout. Some infected ears show brown necrotic lesions at the end of the cob (*Figs. 1A-C*). Often, the decay begins with insect-damaged kernels, by corn borer or bird feeding as a first infection and is later infected by fungi as a secondary infection. Normally, the disease does not involve the whole ear or kernels, but a portion of the corn ear. The infected kernels are frequently appeared as tan or brown, or have white streaks of the fungal mycelia.

The *Fusarium* isolates were single-spored and tentatively identified into 10 species (namely, *F. equiseti*, *F. longipes*, *F. nygamai*, *F. oxysporum*, *F. pseudograminearum*, *F. proliferatum*, *F. semitectum*, *F. solani*, *F. subglutinans*, and *F. verticillioides*) as shown in Table 1. Among the 10 species, *F. proliferatum* (Section Liseola) was the most prevalent species and widely distributed (it was found to be present in seven locations examined with 58 isolates). On the contrary, three species, namely, *F. equiseti*, *F. longipes* and *F. nygamai*, were noted as the least frequent species with a single isolate each. The *Fusarium* species in the Section Gibbosum (*F. equiseti* and *F. longipes*) and Section Arthrosporiella (*F. semitectum*) were also isolated and have previously been reported to be frequently present as saprophytes on various plants including maize (Nelson *et al*., 1983, Summerell *et al*., 2003; Leslie & Summerell, 2006). However, *F. proliferatum, F. verticillioides*, *F. solani* and *F. oxysporum* commonly cause plant diseases on a variety of crops (Summerell *et al*., 2003; Leslie & Summerell, 2006).

The *Fusarium* species associated with the corn samples, showing the typical symptoms of Fusarium ear rot disease in Malaysia, are very

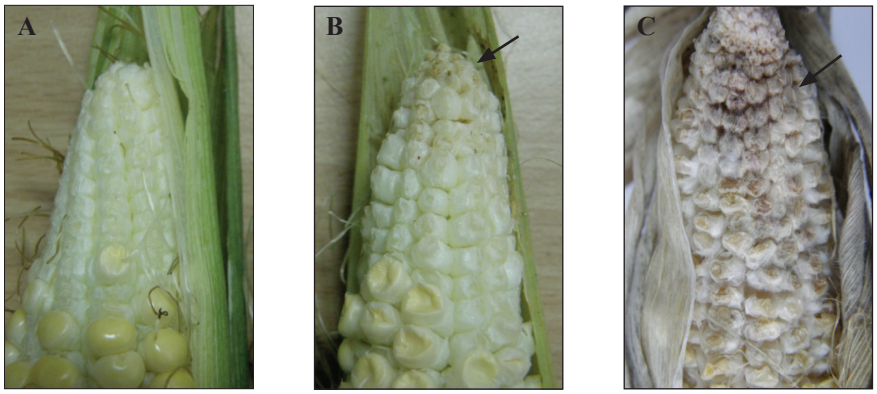

Fig. 1: Samples of the corn obtained from Cameron Highland, Pahang; A) Healthy corn; and B-C) infected cobs showing brown necrotic lesion on the ears (arrows). Scale bar = 0.7cm

diverse, based on Shanon-Weiner Index. The *Fusarium* species isolated from the samples obtained from Semenyih was the highest in term of its diversity, with H'= 1.72, and this was followed by Banting (1.46), TPU UPM and MARDI Pulau Pinang (1.41), Cameron Highland (1.25), Jerantut (0.69), Lanchang (0.51), and Seri Medan (0.46). The lowest index (0) was obtained by the samples from Jengka, Kuala Berang and Kota Kinabalu (Table 1).

The cultures were identified as the *Fusarium* species based on several diagnostic characteristics, such as the presence of chlamydospores, microconidia, macroconidia and the type of conidiophores. The number of macroconidia septation is variable, depending on the species. Meanwhile, *F. equiseti*, *F. longipes*, and *F. pseudograminearum* only produced macroconidia and without microconidia. Most of the species in Section Liseola and their allied (which were tentatively identified as *F. nygamai*, *F. proliferatum*, and *F. verticillioides*) produced microconidia in chains and/or false heads. Another archetypal characteristic of this particular section is that the majority of the species were unable to produce chlamydospores.

The most dominant species was morphologically identified as *F. proliferatum* that belongs to the section Liseola and their allied. This species has a worldwide distribution and is frequently isolated on an economically important plant including maize (Nelson *et al*., 1983; Marasas *et al*., 1984). Oláh *et al*. (2006) state that *F. proliferatum* is a weak pathogen of maize and it enters into host tissues during germination.

The second highest isolate is *F. semitectum* which is regularly found as a secondary invader in diseased tissues (Summerell *et al*., 2003), soils (Burgess *et al*., 1994), as well as from diverse aerial parts of several plants, including maize (Andrés Ares *et al*., 2004), asparagus

TABLE 1
The occurrence and frequency of *Fusarium* species isolated from corns in Malaysia

Fusarium species	Total (number of isolates)	Main corn growing areas											
		Jerantut, Pahang	Jengka, Pahang	Lanchang, Pahang	Cameron Highland, Pahang	Banting, Selangor	Semenyih, Selangor	TPU, UPM, Selangor	Seri Medan, Johor	Senggarang, Johor	Kuala Berang, Terengganu	MARDI, Pulau Pinang	Kota Kinabalu, Sabah
F. equiseti	1	-	-	-	1	-	-	-	-	-	-	-	-
F. longipes	1	-	1	-	-	-	-	-	-	-	-	-	-
F. nygamai	1	-	-	-	-	-	1	-	-	-	-	-	-
F. oxysporum	20	-	-	-	10	3	3	2	-	-	-	2	-
F. pseudograminearum	19	-	-	-	19	-	-	-	-	-	-	-	-
F. proliferatum	58	-	-	3	-	2	6	21	5	3	-	18	-
F. semitectum	47	-	-	-	4	1	9	18	-	1	-	6	8
F. solani	15	1	-	-	-	3	9	-	-	-	-	2	-
F. subglutinans	34	1	-	11	1	6	5	8	-	-	1	1	-
F. verticillioides	24	-	-	-	1	-	1	11	1	3	-	7	-
Percentage (%)	-	0.9	0.4	6.4	16.4	6.8	15.5	27.3	2.7	3.2	0.4	16.4	3.6
Total (n)	220	2	1	14	36	15	34	60	6	7	1	36	8
Shannon-Weiner Index	-	0.69	0.0	0.51	1.25	1.46	1.72	1.41	0.46	1.0	0.0	1.41	0.0

(Al-Amodi, 2006), kangaroo paw (Satou *et al*., 2001), beans (Dhingra & Muchovej, 1979), sorghum (Gopinath *et al*., 1985), millet (Mathur *et al*., 1973; 1975) and potatoes (Kim *et al*., 1995). Meanwhile, *F. verticillioides* has been reported as a pathogen on maize which causes epidemics of maize ear rot. The species can be found worldwide, or wherever maize is cultivated (Leslie & Summerell, 2006).

Three species (namely, *F. equiseti*, *F. nygamai*, and *F. longipes*) were also isolated from the maize samples showing typical symptoms of Fusarium ear rot disease which were previously recovered, particularly from diverse hosts. These species are cosmopolitan soil inhabitants that have been recovered from many parts of the world, primarily as saprophytes or endophytes (Nelson *et al*., 1983; Summerell *et al*., 2003; Leslie & Summerell, 2006) and have also been isolated from maize (Logrieco *et al*., 1998). Similarly, Logrieco *et al*. (1998) have reported that those species produce beauvericin and may be one of the contaminants of maize.

A total of 14 isolates of *F. pseudograminearum* were obtained from Cameron Highlands in Pahang, whereby the day and night temperature range is 18-25°C. Based on the colony growth requirement, this species is categorized as a low temperature tolerates fungus, and therefore, the species is usually found in the temperate region. Moreover, *F. pseudograminearum* is morphologically and culturally indistinguishable from *F. graminearum,* whereby both species can only be differentiated by observing the formation of perithecia on media such as CLA. *F. graminearum* is homothallic and able to abundantly produce perithecia on the media, although *F. pseudograminearum* is not capable of producing perithecia (Leslie & Summerell, 2006). *F. pseudograminearum* and *F. graminearum* are important plant pathogens worldwide, including the maize disease. Diseases of cereals, including maize, caused by these species are responsible for large economic losses due to the reduction in seed quality and contamination of grain with their secondary metabolites that are known as mycotoxin (Russell *et al*., 2005).

In conclusion, ten species of *Fusarium* were isolated from 12 locations in Malaysia and they were morphologically identified as *F. equiseti*, *F. longipes*, *F. nygamai*, *F. oxysporum*, *F. proliferatum*, *F. pseudograminearum*, *F. semitectum*, *F. solani*, *F. subglutinans*, and *F. verticillioides.* This is a rather comprehensive report on the diversity of the *Fusarium* species associated with corn in Malaysia and the role of these fungi in causing plant diseases; nonetheless, their biological species and toxigenicity still require further investigation.

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α-Tocopherol, Ascorbic Acid and Carotenoid Content in *Centella asiatica* Leaf Tissues and Callus Cultures

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ABSTRACT

Green leafy vegetables constitute a major part of balanced diet and are good sources of minerals and vitamins. These beneficial effects are attributed to the presence of antioxidants. Antioxidants also contribute to the defence mechanisms against oxidative stress. *Centella asiatica*, which is locally known as 'pegaga,' is claimed to be rich in natural antioxidative compounds. This study was conducted to determine the amount of ascorbic acid, α-tocopherol and carotenoid content in twelve accessions of *C. asiatica* (CA01 to CA12-comprises of 'pegaga nyonya', 'pegaga kampung' and 'pegaga salad') leaf tissues and callus cultures. The antioxidative constituents of *C. asiatica* in the leaf tissues and cultures were found to vary significantly between the accessions. In particular, CA03 leaves ('pegaga salad') exhibited the highest concentrations of ascorbic acid (95.86 ± 12.60 mg/g.fwt), whereas CA10 ('pegaga nyonya') produced the highest concentration of α-tocopherol (0.233 ± 0.029 µg/g.fwt) and carotenoids (36.55 ± 0.06 mg/g.fwt). The antioxidants studied were also successfully detected in the cultures of *C. asiatica*, with CA08 callus ('pegaga kampung') being dominant in ascorbic acid (167.21 ± 5.30 mg/g.fwt) and α-tocopherol (5.72 ± 0.29 µg/g.fwt), whereas CA12 callus ('pegaga kampung') had the highest carotenoid content (1.04 ± 0.07 mg/g.fwt). Meanwhile, the amount of non-enzymatic antioxidants (except for carotenoid content) was significantly higher in the cell cultures compared to the leaf tissues. The results indicated that CA03 and CA10 leaf tissues, as well as CA08 and CA12 calli were good sources of natural antioxidants compared to other accessions.

Keywords: *Centella asiatica*, leaf tissues, callus cultures, antioxidants, oxidative stress

INTRODUCTION

Antioxidants are secondary constituents and can be defined as anything that can inhibit or prevent oxidation of a susceptible substrate. Plants produce a wide array of antioxidant compounds which include carotenoids, ascorbic acid, tocopherols, and tocotrienols (Hollman, 2001). These plant-based dietary antioxidants are believed to have important role in the maintenance of human health due to its ability to provide protection or defence mechanism against constant and unavoidable challenges of reactive oxygen species (ROS) (Fridovich,

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1998). The formation of ROS in plants creates a condition called oxidative stress that can damage cellular components, and thus, plants have protective mechanisms to prevent or in defence from oxidative damages (Apel & Hirt, 2004). Meanwhile, the toxic effects of ROS are counteracted by enzymatic and non-enzymatic antioxidative systems, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), ascorbic acid, tocopherol, glutathione, and phenolic compounds (Ahmad *et al.*, 2008).

Centella asiatica, which is locally known as 'pegaga', belongs to the family Apiaceae; it is widely found in the tropical and subtropical regions. In addition, *C. asiatica* is commonly consumed as vegetable ('ulam/salad') among the Malays, as a cooling drink by the Chinese and as a brain tonic by the Indians. Moreover, pegaga is claimed to possess a wide range of beneficial effects, and it is treated as a valuable medicinal plant in the Chinese traditional medicine and classical Indian Ayurvedic medicine (Peiris & Kays, 1996). It is also used in the treatment of various skin diseases (Patra *et al.*, 1998), as healing properties (Suguna *et al.*, 1996), anticancer property (Babu *et al.*, 1995), antioxidant property (Zainol *et al.*, 2003) and antileprotic property (Sahu *et al.*, 1989).

To date, relevant research has focused on the production of triterpenoids and phenolic content, as well as the antioxidative properties of the plant. However, studies on the production of antioxidants as defence strategies, particularly vitamin and enzymatic antioxidants from this plant, is still very limited. Therefore, this study was carried out to evaluate the production of ascorbic acid, α-tocopherol, and carotenoid content of the leaf tissues and leaves-derived callus of *C. asiatica*.

MATERIALS AND METHODS

Plant Materials

Twelve accessions of *C. asiatica* (CA01 to CA12, leaves) were obtained from Malaysian Agriculture Research and Development Institute (MARDI), Serdang Selangor.

Callus Initiation and Maintenance

Sterile leaf explants were aseptically cultured on solid Murashige and Skoog (MS) basal medium (Murashige & Skoog, 1962), supplemented with 2.0 mg/l 2,4-D and 1.0 mg/l Kinetin, and 30 g/l sucrose was added as a carbon source and B5 vitamins (Gamborg *et al*., 1968). Meanwhile, Gelrite agar (2.5 g/l) was used to solidify the culture medium. The cultures were maintained by regular sub-culturing at 10 days interval on fresh medium. All the cultures were incubated in 12h/12h (light/dark) photoperiod under cool, white fluorescent lamps at 27 ± 2°C. Friable calli obtained were used for the antioxidant assays.

Antioxidant Assays

α-Tocopherol was extracted based on the method by Hodges *et al*. (1996) and the assay mixture was prepared as described by Kanno & Yamauchi (1977). A standard curve was prepared using α-tocopherol (Sigma, type V) at various concentrations (0-1.4 µg/ml). The amount of α-tocopherol in the leaf samples was calculated based on the standard curve. Ascorbate was extracted according to the procedure of Jagota and Dani (1982). Absorbance of the mixture was measured at 760 nm. A standard curve was prepared using ascorbic acid at various concentrations (0-60 µg/ml), and the amount of ascorbic acid was calculated based on the standard curve. Carotenoid content was analyzed according to the method proposed by Lichtenthaler (1987). Supernatant of the leaf samples was measured spectrophotometrically at 663.2, 646.8 and 470nm, while 80% acetone was used as a blank.

RESULTS AND DISCUSSION

The ascorbic acid content in the leaf tissues varied from 27.35 ± 2.33 to 95.86 ± 12.60 mg/g. fwt (*Fig. 1A*). The high productions of ascorbic acid in this study were paralleled with the previous report that *C. asiatica* leaves are rich in carotenoids, vitamin B and C (Paramageetham *et al*., 2004). However, Chanwitheesuk *et al.* (2005) reported much lower content of ascorbic

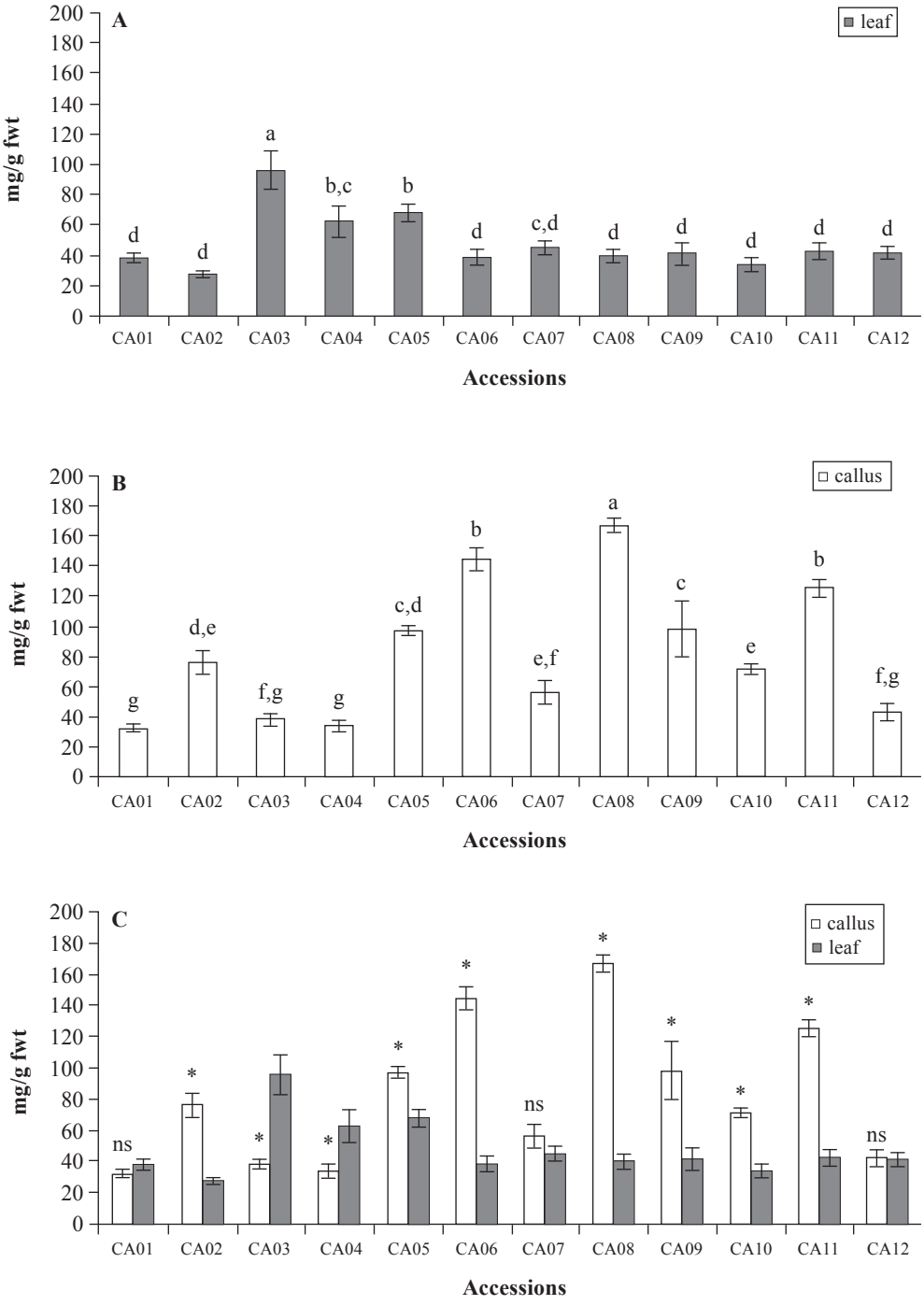

Fig. 1: Ascorbic acid concentrations of C. asiatica*: (A) leaf tissues (B) callus cultures and (C) comparison of leaf tissues and callus cultures. Data shown are means ± SE (n=5). Means with the same letters are not significantly different. * = significantly different, and ns = not significantly different at p<0.05*

acid for *C. asiatica* was cultivated in Thailand, i.e. 6.56 ± 0.10 mg/g dry weight. The variation in the ascorbic acid in this study might be related to the differences in the accessions of *C. asiatica* used. Meanwhile, the variability in ascorbic acid content was also observed in 50 different accessions of broccoli (*Brassica oleracea*), ranging from 0.54 to 1.19 mg/g. fwt. This diversity indicates that potential health benefits depend greatly on the genotype consumed (Kurilich *et al.*, 1999; Vallejo *et al*., 2002). Environmental conditions might also contribute to the alteration of the ascorbic acid concentrations, as reported by Howard *et al*. (1999) in Brasicca vegetables. They observed that the ascorbic acid in plants harvested in the raining season differed than those harvested during high air temperatures. Another factor that might influence the ascorbate pool is light intensities as higher ascorbate was found in the leaves of *Vinca major* and *Schefflera arboricola* grown at high light intensities of 1200 µmol photons $m^{-2}s^{-1}$ (Grace & Logan, 1996; Smirnoff & Pallanca, 1996) and decreases in *Arabidopsis thaliana* and barley leaves during the dark periods (Conklin *et al.*, 1997).

This study revealed that highest concentration of ascorbic acid was detected in CA03 leaf (95.86 ± 12.60 mg/g.fwt). This might be due to the unique characteristics possessed by CA03. Morphological studies by Anna (2004) revealed that the leaf of CA03 is lighter green in colour compared to deep green for all other accessions; the only accession that possesses lobed and crispate leaf margin and also represents the furthest relationship compared to the entire accessions based on the dendogram of genetic distance. Wong (2003) also found that CA03 represented the furthest relationship among the accessions, with 15% of polymorphism with the Amplified Fragment Length Polymorphism (AFLP) analysis.

According to Dietary Reference Intakes and the recommended Dietary Allowances (2001), the current RDA for vitamin C is 15-120 mg/ day depending on the gender and age. Taking into account the quantities of vitamin C found in *C. asiatica*, it becomes obvious that by taking only a portion of *C. asiatica* leaves, one can adequately cover the recommended amount. Large variability of ascorbic acid content (ranging between 32.43 ± 3.09 to 167.21 ± 5.30 mg/g. fwt as shown in *Fig. 1B*) in this study was also observed in the calli. The observation in this study is in agreement with that of Federici *et al*. (2003) who found that the variability observed within the strain collection cultivated under the same culture conditions might be due to the somaclonal variation induced during the initiation of cultures. Successful production of ascorbic acid has also been reported in the two cell lines of *Helianthus annuus* L (Caretto *et al.*, 2002) and in root cultures of *Panax ginseng* and *P. quinquefolium* (Ali *et al.*, 2005).

The α-tocopherol concentrations in *C. asiatica* varied from 0.065 ± 0.001 to 0.233 ± 0.029 µg/g.fwt. The highest amount was found in CA10 leaf. A high content of α-tocopherol was also present in CA01, CA09 and CA02, while other accessions showed lower α-tocopherol concentration (*Fig. 2A*). A higher concentration of α-tocopherol (31 ± 5 µg/g.fwt) was reported in the same plant originated from Thailand (Chanwitheesuk *et al.*, 2005). The concentration of α-tocopherol in this study was also significantly lower compared to the content of α-tocopherol reported by Ching & Suhaila (2001) in *Hydrocotyle asiatica* (29.8 ± 2.2 µg/g edible portion). They also reported that the α-tocopherol content in 62 edible tropical plants, including 3 commonly eaten leafy vegetables or *'ulam'* per 100g fresh weight were 42.68 ± 0.12 mg (*Sauropus androgynus;* cekur manis), 14.68 ± 0.51 mg (*Oenanthe javanica*; selom) and 5.97 ± 0.21 (*Piper sarmentosum*; kaduk). The values of α-tocopherol content reported were significantly higher than those found in this study. The variation of α-tocopherol observed in this study is in agreement with the statement by many researchers that the level of tocopherol derivatives may differs quantitatively and qualitatively among different plant species and varieties, and even within a given plant species or within organs. Strong variations in α-tocopherol contents were also found to depend on the developmental stage of the leaves

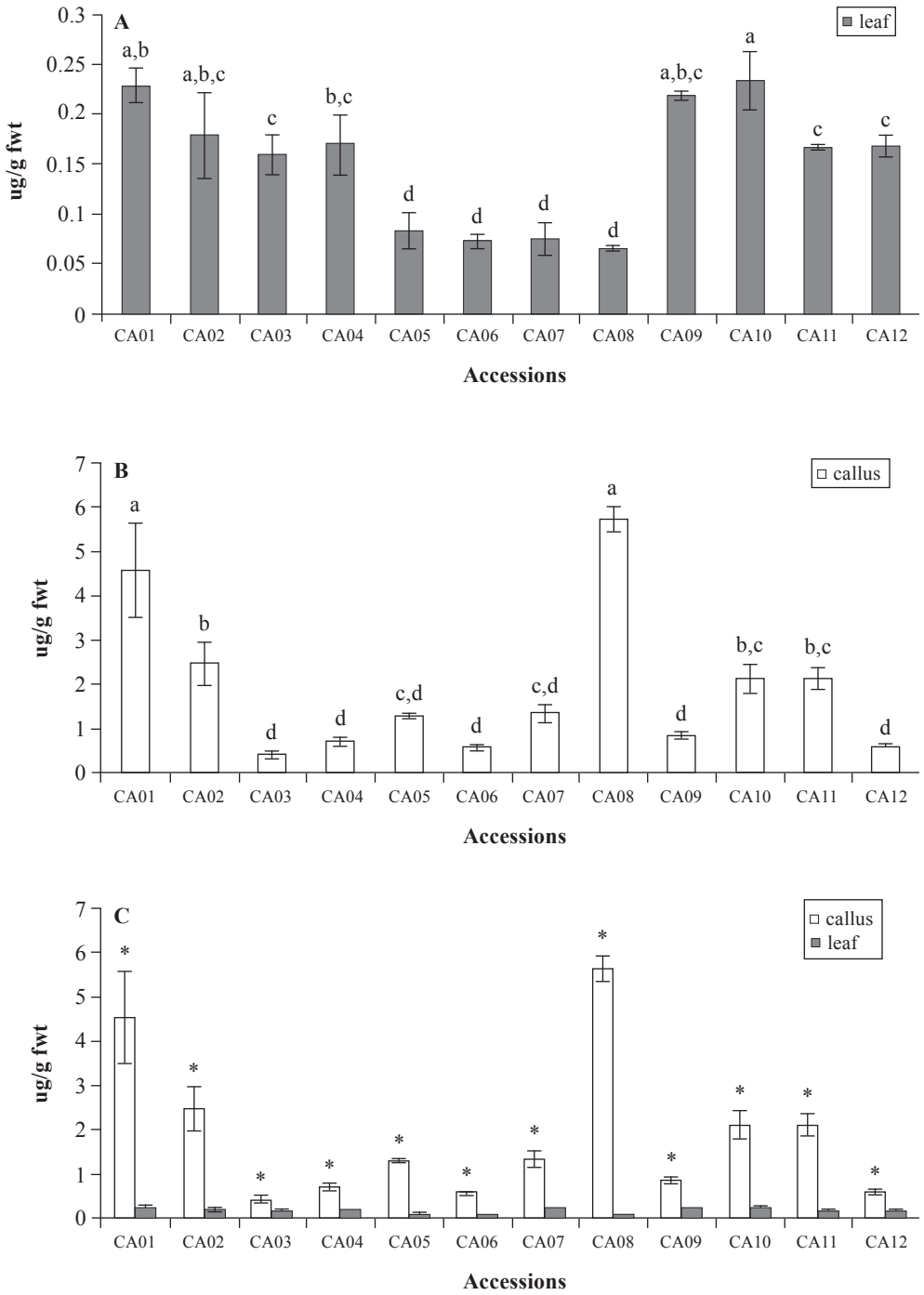

Fig. 2: α-Tocopherol concentrations of C. asiatica*: (A) leaf tissues (B) callus cultures and (C) comparison of leaf tissues and callus cultures. Data shown are means ± SE (n=5). Means with the same letters are not significantly different. * = significantly different and ns = not significantly different at p<0.05*

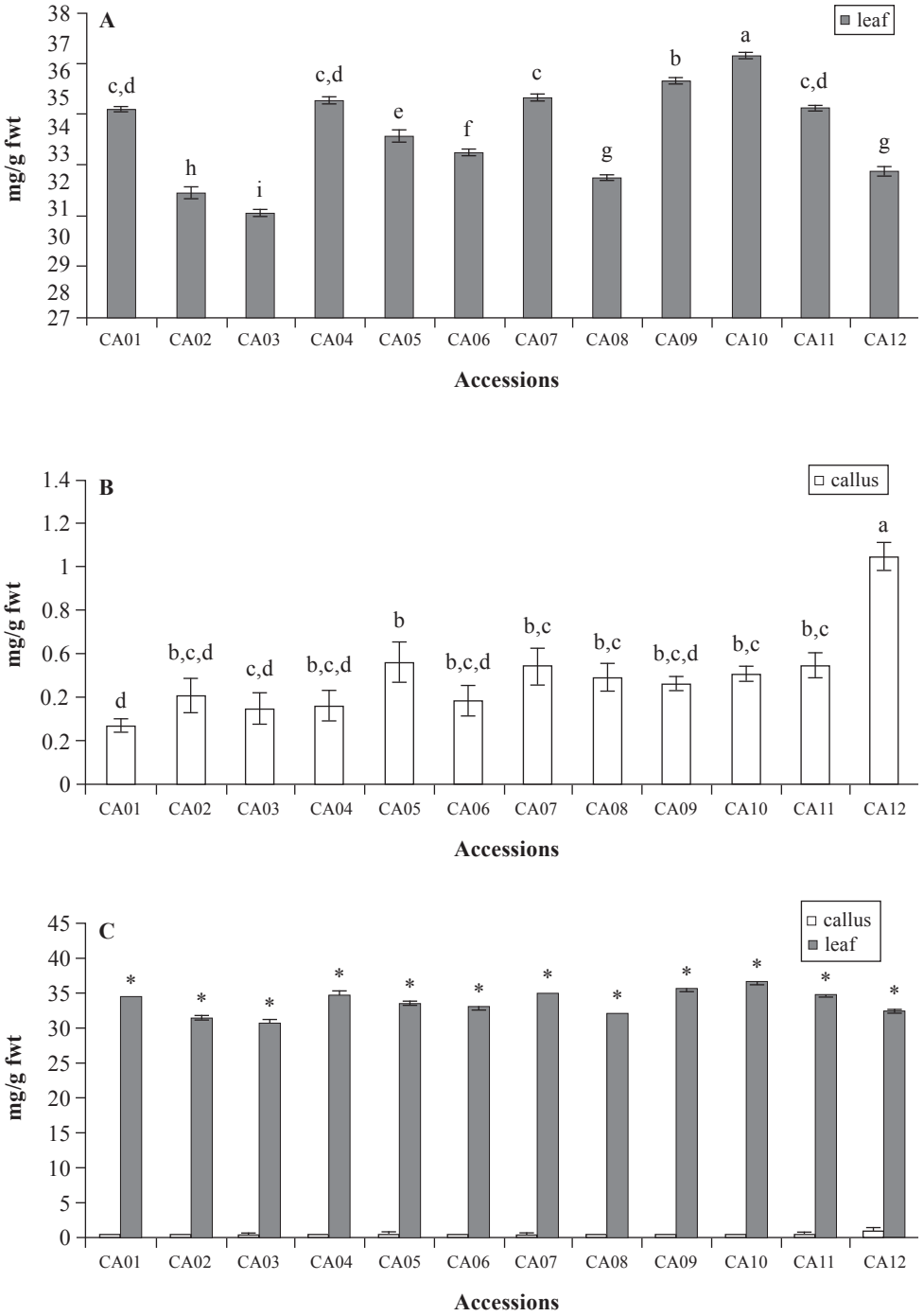

Fig. 3: Carotenoids concentrations of C. asiatica*: (A) leaf tissues, (B) callus cultures, and (C) comparison of the leaf tissues and callus cultures. Data shown are means ± SE (n=5). Means with the same letters are not significantly different. * = significantly different and ns = not significantly different at p<0.05*

and the environmental conditions, such as light intensity, temperature, drought and pollutants (Munne-Bosch & Alegre, 2000). Similarly, *C. asiatica* calli were also identified for their differing capability to produce tocopherols (*Fig. 2B*). α-Tocopherol concentration was dominant in CA08 (5.72 ± 0.29 µg/g. fwt), followed by CA01. Lower concentrations of α-tocopherol were observed in other accessions. The production of α-tocopherol in this study was significantly lower (24 µg/g. fwt) than the value reported in sunflower cell cultures (Caretto *et al.*, 2004) and of 5-13 mg/100 g dry weight in the cell culture of *Carthamus tinctorius* (Furuya *et al*., 1987).

The amount of carotenoid concentration detected in *C. asiatica* was higher than 22 species of vegetables studied by Muller (1997), while Podsedek (2007) reported lower concentrations of carotenoid (0.26 to 6.1 mg/100 g) in Brussels sprouts, broccoli, red cabbage and white cabbage. Several physical factors, including environmental differences (such as temperature, soil and solar intensity) can affect the total nutrient values in plants. Calli of *C. asiatica* managed to produce low amount of carotenoid content except in CA12 which contained almost 2-folds higher of the total carotenoid compared to other accessions (*Fig. 3*). The carotenoid content of *C. asiatica* cultures was in the range of 0.27 ± 0.03 to 1.04 ± 0.07 mg/g fwt and was significantly lower compared to 5.7 to 13.3 mg/g fwt in the *Morinda elliptica* cell cultures in various medium strategies (Chong *et al*., 2004).

It is interesting to note that the analyses conducted in the present work revealed that the greatest accumulation of almost all antioxidant studied occurs in the callus cultures compared to the leaf tissues (*Figs. 1C* and *2C*), except for carotenoids (*Fig. 3C*), while the concentration of the antioxidant studied varied between the accessions.

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Subchronic Oral Toxicity Study of *Morinda citrifolia* (Mengkudu) in Spraque Dawley Rats

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ABSTRACT

A subchronic oral toxicity study was conducted to evaluate the safety of *Morinda citrifolia* in Sprague-Dawley (SD) rats. For this purpose, the fruit of *Morinda citrifolia* were oven dried and ground into powder form before incorporating into diet and fed to SD rats (10 males and 10 females per group) at dose levels of 2000 (low dose) and 5000 (high dose) mg/kg body weight/day for 13 weeks. Clinical observations were recorded, while body weight and feed consumption were measured throughout the study. At the end of the study, all the rats were subjected to a full necropsy. Their blood samples were collected for clinical pathology, whereas selected organs were weighed and tissues were preserved from all the animals. Total protein was found to be significantly lower ($p<0.05$) in male rats of all the treatment groups. Meanwhile, total white blood cells ($3.96 \times 10^3/\mu l$) and spleen weight (0.14%) were found to be significantly lower ($p<0.05$) in female rats of the low dose group. Nevertheless, the differences observed were within the normal range of normal healthy rats that were considered to be not toxicological significance. It was concluded that the no-observed-adverse-effect level (NOAEL) for *Morinda citrifolia* was 5000 mg/kg body weight/day.

Keywords: *Morinda citrifolia*, oven dried, subchronic, rats, clinical pathology

INTRODUCTION

Morinda citrifolia, which is locally known as "mengkudu", has various names such as "noni" in Hawaii, "Indian mulberry" in Indian subcontinent, "painkiller bush" in the Caribbean and "cheese fruit" in Australia (Nelson, 2001; Ross, 2001; Wang *et al*., 2002). The plant has been used for food and medicinal purposes by Polynesians for more than 2000 years (Chan-Blanco *et al*., 2006). It has also been reported to have assorted therapeutic effects in both human and laboratory animals (Wang *et al*.,

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2002). In view of the large number of medical claims that have been made for its efficacy, the most important thing not to be overlooked for *Morinda citrifolia* is the safety aspect of the plant, especially the fruit.

Morinda citrifolia product, such as the juice, is increasingly popular as a functional drink due to the claim indicating its benefits for many illnesses. Since 1996, worldwide production of noni fruit juice has tremendously increased with more than 80 million litres by French Polynesia alone (European Food Safety Authority, 2006). The increase in the consumption of noni fruit juice has brought about the concern over its safety in people who have been drinking the noni fruit juice product. Thus, various toxicological studies have extensively been carried out on the fruit juice (Tahitian Noni) and it has been reported to be safe by Scantox Biologisk Laboratorioum, Lille, Skensved, Denmark March 2000 and May 2001 (West *et al*., 2006); however, there were reported cases of the toxicity, especially liver toxicity. In the period between 2004 and 2006, there were reported cases published whereby the noni fruit juice was suggested to have been responsible for acute hepatitis (Millonig *et al*., 2005; Staldbauer *et al*., 2005; Yüce *et al*., 2006).

Meanwhile, it is important to note that toxicological evaluation is a need in herbal studies. The health promoting benefits of *Morinda citrifolia* have been known for generations and they are extensively used in many countries for its medicinal properties. The increasing use of this plant has resulted in concerns over both the efficacy and safety of the product. Despite the widespread use of *Morinda citrifolia* in Malaysian traditional medicine, a survey of the literature has indicated a lack of proper toxicological evaluation of these local varieties. Due to the high promising and commercial potential of *Morinda citrifolia* products, it is therefore essential that *Morinda citrifolia* be studied for possible toxicity. It is particularly important in detecting toxicity that occurs either after a short period or after a prolonged exposure to *Morinda citrifolia*. Hence, through the toxicity studies in animals, it is anticipated that it can be viewed to safeguard the public health and ensure greater value for money of the herbal product during normal conditions of use. The toxicity studies have also provided a preclinical safety evaluation standard that is expected to be performed before *Morinda citrifolia* can be evaluated in human. The subchronic oral toxicity study of *Morinda citrifolia* was performed in Sprague-Dawley (SD) rats to provide benchmark data so as to understand its safety without the potential confounding variables associated with many commercial *Morinda citrifolia* products. This study was therefore carried out with the objectives to investigate the safety of *Morinda citrifolia* through a 13-week subchronic toxicity study in the male and female Sprague Dawley rats, as well as to determine the no-observe-adverse-effect level (NOAEL) of *Morinda citrifolia* in rats fed at the doses of 2000 mg/kg and 5000 mg/kg body weight.

MATERIALS AND METHODS

Test Material

Fresh fruits of *Morinda citrifolia* were obtained from MARDI Research Station, located in Muadzam Shah, Pahang. The fruit samples were finely sliced into 8 mm in thickness and dried in an oven at 55 - 60 °C for 48 hours. The samples were then ground into powder, followed by incorporating it into commercial rodent diet that had previously been ground before the mixing process. Finally, the combination of dried fruit samples and commercial rodent diets were given as treatment diets for the toxicity trial. The treatment diets were freshly prepared every week according to the body weight of the rats.

Animals Management

Sixty male and female Sprague-Dawley (SD) rats at 6 weeks of age, with an average body weight of 170 - 200 grams, were used in the study. The rats were acclimatized to the housing conditions for a period of 1 week, and the treatment started at the age of 7 weeks. All the rats were individually housed in a polycarbonate

mesh bottom cage during the acclimatization period and, were thereafter kept in a room maintained at a temperature of 25–27°C and a relative humidity of 40–70% with a 12-h light/ dark cycle. Each cage was provided with a colour-coded card containing rat number and for dose level identification.

Experimental Design

The rats were weighed and randomly assigned into three groups (10 males and 10 females per group), namely, control, low dose and high dose according to the randomized complete block design (RCBD). When placed in the study, the average weights of the female and male rats were around 170-190 and 220-240 grams, respectively. The powdered *Morinda citrifolia* fruit were incorporated into the treatment diets at the dose levels of 2000 and 5000 mg/kg body weight/ day for 13 weeks. Meanwhile, the control group received commercial rodent diet only. Feed were given *ad libitum* and all rats had free access to water. The amounts of supplied and residual diet were weighed twice a week (at days 6 and 7) in order to calculate the daily feed consumption. The rats were observed daily for clinical signs and mortality, and were weighed weekly. The animals were let to fast overnight prior to necropsy. At the end of 13 weeks or 91-day test periods, all the rats were euthanatized using overdosed diethyl ether. Upon necropsy, their blood samples were taken via caudal vena cava for clinical pathology (clinical biochemistry and haematology), while selected organs were weighed and specific tissues from all animals were preserved for subsequent histopathology examination. The study was conducted at the Animal House, Malaysia Agricultural Research and Development Institute (MARDI) in Serdang, Malaysia, and was complied with the OECD Guidelines for the Testing of Chemicals (OECD Guidelines 408, 1998).

Clinical Pathology

The serum was analysed for alanine amino tranferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), creatinine kinase (CK), urea, creatinine, total protein (TP) and albumin using automated clinical chemistry analyzer (TRX 7010, Biorex Mannheim, Germany). The following haematological components were analyzed: total white blood cells (WBC), red blood cells (RBC), haematocrit/packed cell volume (HCT/PCV), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and platelets, using an automated blood analyzer (Cell Dyn® 3700, Abbott Diagnostic, USA).

Pathology

The main organs, such as liver, kidney, spleen, heart, lungs and testes, were quickly excised for all the excess tissues and immediately weighed after rinsing them in 0.9% cold saline to remove any blood. The organ relative weight (% of body weight) was obtained by dividing the final weight of the organ to the final body weight. The tissue samples of the liver and kidney were routinely processed for histopathology and they were examined under light microscope.

Statistical Analysis

The mean values and standard errors were calculated from the data obtained, and these were then statistically analyzed using SAS version 9.1. Meanwhile, Duncan's multiple range analysis was employed to determine the differences in the parameters of the two sexes and between the treatment groups.

RESULTS

Body Weight

The mean body weights of male and female rats for all the treatment groups are shown in *Figs. 1* and *2*. There was no significant difference observed that was attributed to the administration of the test substance between sexes. There was no difference between the treatment groups in

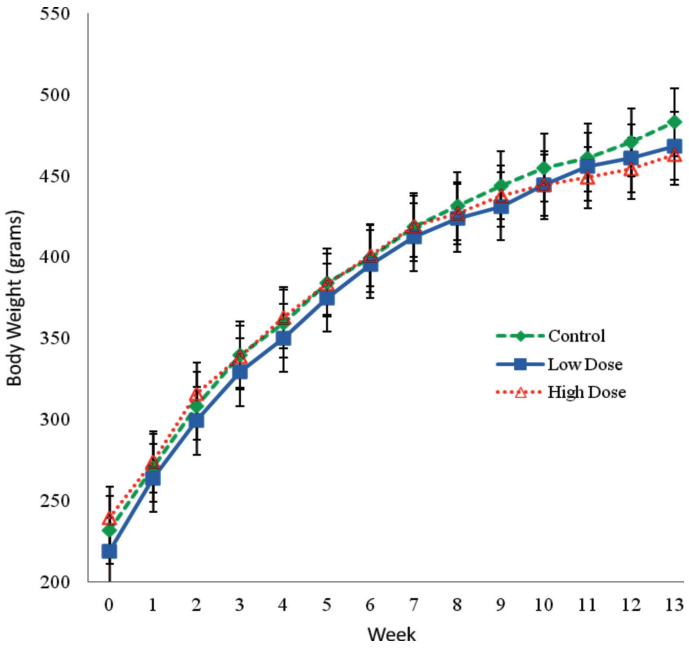

Fig. 1: The mean body weights of the male rats that were given Morinda citrifolia *incorporated in their diet for three months*

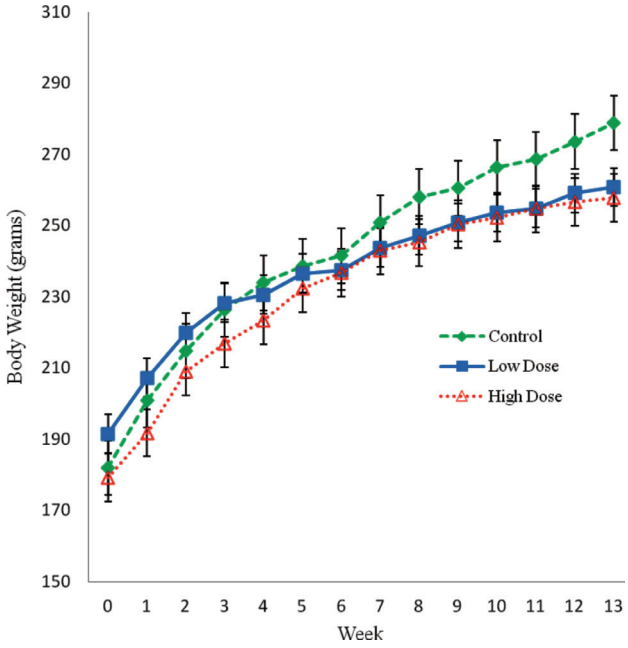

Fig. 2: The mean body weights of the female rats given Morinda citrifolia *incorporated in their diet for three months*

the male rats throughout the experimental period, except at week 2, whereby the body weight was significantly ($p<0.05$) lower in the low dose group (*Fig. 1*). In female rats, the mean body weight was significantly ($p<0.05$) lower at week 12 in the high dose group and in both the low and high dose groups at week 13, as compared to the control (*Fig. 2*).

Clinical Pathology

Generally, there was no significant difference between the sexes in all the parameters measured for haematology (Table 1) and biochemistry (Table 2). Some statistically significant changes between the treatment groups in certain haematology and biochemistry values were also noted. Nevertheless, the magnitudes of the changes were not biologically relevant and the values were also within the normal range of rats of this strain and age. The white blood cells (WBC) count (Table 1) of the females in the low dose group were significantly ($p<0.05$) lower compared to the control and high dose groups. Meanwhile, the total protein (TP) concentration was significantly ($p<0.05$) lower in the male rats as compared to the females in all groups.

Organ Relative Weight

The mean organ relative weights for both the male and female rats in all the treatment groups are shown in Table 3. The spleen weight of the females in the low dose group were significantly ($p<0.05$) lower as compared to the control and high dose groups.

Histopathology

The histopathological examinations of the liver and kidney revealed no significant findings related to the treatments.

TABLE 1
The means haematology values of the treatment groups in the male and female rats
(mean ± S.E.M)

Parameters	Treatment group					
	Control		Low dose		High dose	
	Male	Female	Male	Female	Male	Female
WBC ($\times10^3/\mu l$)	9.78 ± 0.66	5.96[a] ± 0.58	7.95 ± 0.98	3.96[b] ± 0.53	8.78 ± 0.82	4.82[a] ± 0.29
RBC ($\times10^6/\mu l$)	8.85 ± 0.11	7.62 ± 0.17	8.90 ± 0.19	6.92 ± 0.78	8.90 ± 0.11	7.59 ± 0.15
Hb (g/dl)	16.1 ± 0.17	14.4 ± 0.24	16.2 ± 0.34	12.5 ± 1.53	16.1 ± 0.14	14.2 ± 0.27
HCT (%)	50.0 ± 1.24	43.8 ± 1.07	46.9 ± 0.93	37.4 ± 4.23	48.8 ± 1.38	43.4 ± 0.95
MCH (pg)	18.2 ± 0.14	19.0 ± 0.15	18.0 ± 0.10	23.7 ± 6.95	17.9 ± 0.22	18.7 ± 0.15
MCHC (g/dl)	32.4 ± 0.90	33.2 ± 0.72	34.6 ± 0.23	29.9 ± 3.54	33.2 ± 0.86	32.9 ± 0.14
MCV (fl)	56.6 ± 1.56	57.6 ± 1.52	52.1 ± 0.46	48.7 ± 5.55	54.3 ± 1.66	57.4 ± 1.78
PLT ($\times10^3/\mu l$)	1324 ± 25.0	1271 ± 30.9	1298 ± 40.1	1110 ± 42.2	1345 ± 29.8	1350 ± 43.0

[a,b]: Means with different superscript/s within the same row differ significantly ($p< 0.05$)

TABLE 2
The means serum biochemistry values of the treatment groups in the male and female rats (mean ± S.E.M)

Parameters	Treatment					
	Control		Low dose		High dose	
	Male	Female	Male	Female	Male	Female
ALT (U/L)	29.0 ± 0.6	31.2 ± 0.3	26.7 ± 0.5	30.0 ± 0.3	32.7 ± 0.7	31.5 ± 0.4
AST (U/L)	150.7 ± 15.6	149.4 ± 15.0	139.2 ± 11.6	139.2 ± 11.6	132.1 ± 6.2	126.1 ± 11.8
ALP (U/L)	78.6 ± 3.6	63.6 ± 9.7	77.4 ± 4.2	63.4 ± 8.1	89.6 ± 7.1	66.5 ± 9.5
GGT (U/L)	51.3 ± 15.8	23.0 ± 7.2	47.9 ± 11.7	35.0 ± 16.7	67.9 ± 21.0	74.5 ± 4.6
Creatinine (μmol/L)	24.2 ± 15.3	35.9 ± 13.4	16.9 ± 40.2	31.2 ± 12.6	41.5 ± 14.6	40.2 ± 14.2
Urea (mmol/L)	7.5 ± 0.6	6.1 ± 0.5	5.7 ± 0.5	5.8 ± 0.6	6.3 ± 0.8	6.2 ± 0.3
CK (U/L)	197.3 ± 65.1	169.3 ± 61.4	249.0 ± 68.5	177.0 ± 35.1	173.1 ± 51.2	131.6 ± 9.93
TP (g/L)	64.7^{b} ± 1.3	72.9^{a} ± 1.6	65.2^{b} ± 1.2	74.1^{a} ± 1.2	67.9^{b} ± 2.1	74.5^{a} ± 4.6
Albumin (G/L)	40.8 ± 1.2	34.2 ± 0.88	37.3 ± 4.82	34.9 ± 0.43	41.2 ± 2.77	35.9 ± 1.08
Globulin (G/L)	32.0 ± 0.68	30.5 ± 0.92	36.8 ± 4.93	30.0 ± 0.62	33.4 ± 1.88	32.1 ± 1.18
A/G ratio (G/L)	1.3 ± 0.04	1.1 ± 0.04	1.2 ± 0.16	1.2 ± 0.07	1.2 ± 0.02	1.2 ± 0.05

^{a,b}: Means with different superscript/s within the same row differ significantly ($p < 0.05$)

DISCUSSION

In the recent years, there has been growing interest in the safety of herbal products due to a large increase in its consumption as dietary supplements, either for enhancing health or physical performances. The use of complementary and alternative medicine is rapidly increasing in developed countries and in many parts of the world, while uses of traditional medicine remain widespread in developing countries. In Malaysia, safety information with regards to herbs is very limited because there is no universal regulatory system that ensures the safety of phytopharmaceuticals or herbal products (Mohammed, 2006). Most people believe that herbal medicines have no side effects or any potential risks due to their natural origins and are often considered as food supplements, not drugs. This study focused on the herbal plant of *Morinda citrifolia* (locally known as 'mengkudu') which has extensively been used in many countries for its medicinal properties. A number of *in vitro* and *in vivo* studies demonstrate a range of potentially beneficial effects, such as antioxidant and immunomodulatory properties (Olivier & Matthias, 2007). Meanwhile, the increased use of this plant has resulted in concerns over both the efficacy and safety of the product.

TABLE 3
The means organ relative weight of the treatment groups in the male and female rats (mean ± S.E.M)

Parameters	Treatment group					
	Control		Low dose		High dose	
	Male	Female	Male	Female	Male	Female
Body weight	490.4 ± 37.4	265.1 ± 12.1	454.6 ± 14.7	219.1 ± 25.2	477.9 ± 28.6	248.7 ± 6.5
Liver	2.43 ± 0.07	2.75 ± 0.08	2.57 ± 0.08	2.48 ± 0.28	2.48 ± 0.06	2.77 ± 0.10
Kidney	0.56 ± 0.01	0.61 ± 0.02	0.60 ± 0.01	0.54 ± 0.06	0.56 ± 0.02	0.61 ± 0.02
Lungs	0.41 ± 0.03	0.53 ± 0.01	0.38 ± 0.02	0.47 ± 0.05	0.39 ± 0.02	0.57 ± 0.03
Spleen	0.15 ± 0.01	0.18^{a} ± 0.01	0.15 ± 0.01	0.14^{b} ± 0.02	0.15 ± 0.01	0.19^{a} ± 0.01
Heart	0.26 ± 0.01	0.31 ± 0.01	0.26 ± 0.01	0.32 ± 0.03	0.27 ± 0.01	0.32 ± 0.01
Testis	0.58 ± 0.05	n.a	0.53 ± 0.04	n.a	0.61 ± 0.06	n.a

^{a,b}: Means with different superscript/s within the same row differ significantly ($p< 0.05$)

The present study was conducted to evaluate the safety use of *Morinda citrifolia* that was incorporated daily in the diet of male and female Sprague-Dawley rats. As indicated earlier, no deaths and clinical signs of toxicity were observed throughout the experimental period. The consumption of *Morinda citrifolia* was tolerated well and did not produce any general organ or systemic toxicity when fed to the male and female rats at the dose levels of 2000 and 5000 mg/kg/day. One of the indicators for the health status of the experimental animals is an increment in the body weight (Heywood, 1983). In this study, all the rats of both sexes in the treatment and control groups were increased in body weight as they were in the growing stage. At the initial stage of the study, the body weights of the male rats in the low dose group slightly decreased, which could be explained by the attempt of the rats in this group to adjust with the new diet. The decrease in the body weight of the female rats at the end of the experimental period in both the treatment groups as compared to the control could not be determined since the rats were physically healthy and did not show any signs of toxicity. However, the recorded body weights were acceptable for the female rats of this strain at this age. In fact, there were no adverse haematologic effects related to the treatment doses. The cause for the lower WBC in the females of low dose group could not be ascertained since the value was within the normal range of rats of this particular strain and age. In general, clinically low WBC could probably be due to chronic infection, i.e. either bacterial or viral infections. In this study, significantly decreased WBC count was contributed by the decrease in lymphocytes ($2.47 \times 10^3/\mu l$) [data not shown], however these were still within the normal range ($1.64 – 19.5 \times 10^3/\mu l$) of normal healthy rats. Lymphocytes are a type of white blood cell that is responsible for protecting the body against bacterial and viral infections. One of the most common causes of clinically low WBC is an underlying viral infection which can cause a temporary drop in lymphocytes as more

of them are drawn away to fight the infection (David, 2000). Based on the observation made throughout the experimental period, all the rats were clinically healthy and did not show any signs of infection. No treatment-related changes were found in the serum biochemistry of the male and female rats in all the groups. Biochemical measurements, indicative of liver injury such as ALT, AST, ALP, GGT, and total protein, revealed no treatment-related effects. The periportal area of the liver is the first area of the hepatic lobule to be exposed to a toxin being delivered *via* the blood (Huxtable, 1988) and there were no treatment-related changes of the liver enzymes in the male and female rats. The cause for the lower TP in the males of all groups could be explained by the physiological fact since the values were within the normal ranges (60.0–67.0 G/L) for healthy male SD rats. The parameters indicative of kidney functions, such as urea and creatinine (Moshi *et al*., 2001) also showed no treatment-related effects. Organ relative weight measurement is another important guide to assess general toxicity. In particular, changes in the organ weight are indicators of toxicity since these will be affected by the suppression of the body weight (Heywood, 1983). Although the weight of female spleen in the low dose group was significantly lower, the magnitude of the difference is rather small and comparable to the weight of the male spleen in the control and treatment groups, and thus, this cannot be considered clinically relevant. Based on the literature review of the phytochemical content of *Morinda citrifolia*, the histopathological examination was only done on the liver and kidney since these are the two most important organs for detoxification process in the body. The histopathological examination was conducted in all the animals in the control and high dose groups. In the rats that received high doses of *Morinda citrifolia*, no microscopic lesions attributable to the treatment were observed. More importantly, the no treatment-related changes observed in any of the dose groups for the histological examinations demonstrated the absence of toxicity to the liver and kidney.

CONCLUSION

In this study, *Morinda citrifolia* did not show any toxicity even when it was fed at a high dose of 5000 mg/kg body weight. The minor effects observed in both the males and females of the low dose group did not appear to be of toxicological significance. In conclusion, if *Morinda citrifolia* is consumed at the rate of 5000 mg/kg body weight/day, there is likely no chance of developing toxicity, as proven in this study. Therefore, the NOAEL for *Morinda citrifolia* was determined to be greater than 5000 mg/kg body weight per day.

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Histopathological Features of Peripheral T-cell Lymphoma in Sprague Dawley Rats Induced with *N*-methyl-*N*-nitrosourea

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ABSTRACT

This study described the histopathological features of peripheral T-cell lymphoma in male Sprague Dawley rats following intraperitoneal (i.p.) injections of *N*-methyl-*N*-nitrosourea (MNU) at a dose of 60 mg/kg body weight per injection, administered twice weekly for 2 consecutive weeks, and followed by a five-month's observation period. Control rats were injected with normal saline, i.p. All the rats treated with MNU had enlargement of lymph nodes, with 30% had hepatosplenomegaly and 7% had enlarged kidneys at necropsy. Malignant lymphoma was observed in the lymph nodes, spleen, liver, lung, heart, and kidneys. The neoplastic cells were characterised as undifferentiated, and small to large size with bizarre pleomorphic nuclei. The severity was further described as mild, moderate and severe, based on the diffuseness of the lesions. Nonetheless, similar lesions were not observed in the thymus of the rats. Immunohistochemistry staining of the organs was positive for CD3 antibody, which is consistent with T-cell lymphoma.

Keywords: *N*-methyl-*N*-Nitrosourea (MNU), rats, peripheral T-cell lymphoma

INTRODUCTION

Development of animal model for human lymphoma may provide a basis for investigating new biomarkers for early diagnosis and new anti-tumour therapies. Studies have shown that *N*-methyl-*N*-nitrosourea (MNU) induced lymphoma and leukaemia in rats (Koestner *et al*., 1977; Mizoguchi *et al*., 1993; da Silva Franchi *et al*., 2003; Hutheyfa *et al*., 2009a, b; Hazilawati *et al*., 2010a, b). The chemical is one of the *N*-nitroso compounds which is found in food and tobacco smoke (Fiddler, 1975; Beranek, 1990). It has a broad spectrum of the target organs including the lympho-haemopoietic system. Our preliminary study, for instance, has shown that this particular chemical induced stages IV and III lymphoma in young adult and adult male Sprague Dawley rats, respectively (Hutheyfa *et al*., 2009a).

Diagnosis of lymphoma is made based on microscopic examination of the lesions. It could be done either through cytological and/

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or histopathological examinations. Meanwhile, the final diagnosis of lymphoma could only be achieved through histological examination. The final diagnosis of disseminated lymphoma, which is mostly seen in cats (Hazilawati, unpublished), however, could be made through cytological evaluation of the fluid samples which were obtained from pleural cavity of the cats. In cases of generalised canine lymphosarcoma, confirmatory diagnosis could also be made cytologically, provided that an adequate number of malignant lymphocytes from the enlarged lymph nodes were aspirated (Hazilawati, unpublished). Cytological diagnosis of lymphoma in human and animals is always challenging, if adequate cells could not be obtained, which warrants a biopsy for histological evaluations. The objective of this study is to describe the pathological features of peripheral T-cell lymphoma in Sprague Dawley rats induced with MNU.

MATERIALS AND METHODS

Animals

Thirty-two 6-week-old male Sprague Dawley rats, with body weight ranging from 150 to 180 g, were obtained from the Laboratory Animal Resource Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia (UKM) in Bangi, Selangor. The rats were placed in polypropylene plastic cages (two per cage) which were bedded with commercialised wood chips, and housed in a colony animal room with controlled conditions at the temperatures of 22-27°C, 40-70% humidity and 12-hours of light/dark lighting in the Animal House, at the Malaysian Agricultural Research and Development Institute (MARDI) in Serdang, Selangor. The rats were acclimatised for two weeks and fed with commercial rodent chows and drinking water ad *libitum* on a daily basis.

Chemical Carcinogen

N-methyl-*N*-nitrosourea (MNU) (Sigma-Aldrich®, N4766-25G) was used to initiate the carcinogenesis process. The chemical solution was freshly prepared by dissolving 14 mg of MNU in 1 mL of normal saline (pH 4.5).

Experimental Design

The rats were weighted and divided into two groups, with sixteen rats in each, after two weeks of acclimatisation. The MNU group received four intraperitoneal (i.p) injections, twice weekly for two consecutive weeks of MNU at a dose of 60 mg/kg of the body weight (the total dose received was 240 mg/kg of the body weight). Meanwhile, the control group was injected with normal saline, i.p. The rats were humanely sacrificed after 20 weeks of the experimental period by abdominal bleeding under anaesthesia with xylazine (100 mg/kg) and ketamine (40 mg/kg).

Organ Samples

Gross examination for the presence of tumor masses, and enlargement of lymph nodes and internal organs was performed at necropsy. Spleen, liver, lung, kidneys, thymus, and heart were immediately removed and blotted dry.

Histopathology

The necropsied samples were washed with cold normal saline and fixed in 10% formal buffered formalin for at least 48 hours. After fixation, the samples were trimmed at 5 mm thickness and placed in plastic cassettes before they were processed using a standard overnight method in an Automated Tissue Processor (Leica ASP300, Germany). The samples were embedded in paraffin using a Tissue Embedding Console System (Leica EG1160, Germany) via a routine method of paraffin embedding procedure. The tissue samples were sectioned at 4 um thicknesses using a microtome (Leica RM2155, Germany). The tissue sections were placed on water bath (Leica H1210, Germany) at 35°C to 37°C, mounted on glass slides using a hot plate (Leica HI1220, Germany) and stained with Haematoxylin and Eosin (H&E) stain, as described by Luna (1968).

Immunohistochemistry

In this study, the immunohistochemistry analysis was performed using a Dako Envision®+Dual Link System-HRP (DAB+) kit (Dako, USA, Cn: K4965), as per manufacturer's instruction. The kit contains a blocking agent, secondary antibody labelled to horseradish peroxidase and diaminobenzidine [DAB]). The primary antibody (rabbit polyclonal to CD3 primary antibody, ab5690, ABcam®, USA) directed against CD3 (T-lymphocyte marker), was used to classify the lineage of the neoplastic cells in the necropsied organs. The primary antibody was spun in a centrifuge machine (Hettich mikro-120, Germany) at 12000 rpm for 20 seconds before it was diluted at a 1:100 dilution with antibody diluent and background reducing components (Dako, S3022). The paraffinised embedded sample sections for CD3 staining was pre-treated by steaming at 97°C in the target retrieval buffer (Dako, S3001) for 25 minutes. The tissue sections were incubated with anti-CD3 antibody for 30 minutes at room temperature. CD3 immunoreactivity was detected using the labelled polymer-HRP reagent (horseradish peroxidase labeled polymer conjugated to goat anti-rabbit and goat-anti-mouse secondary antibody) and visualised with DAB. All the immunohistochemical sections were counterstained with Mayer's hematoxylin, dehydrated in graded concentrations of ethanol (70%, 90%, and 100%), and cover slipped routinely using permanent mounting medium. The tissue sections were then examined under 10, 20, and 40x magnifications using a light microscope.

RESULTS AND DISCUSSION

N-methyl-*N*-nitrosourea (MNU) is one of carcinogens that has been used for induction of lymphoma and leukaemia (Joshi & Frei, 1970; Koestner *et al*., 1977; Baines *et al*., 1979; Uwagawa *et al*., 1991; Hagiwara *et al*., 1993; Mizoguchi *et al*., 1993; da Silva Franchi *et al*., 2003; Marton *et al*., 2008; Hutheyfa *et al*., 2009a, b; Hazilawati *et al*., 2010a, b), thymomas (Frei,1980), mammary gland (Esendagli *et al*., 2009), prostate (McCormick *et al*., 1999) and gastrointestinal (Mizoguchi *et al*., 1993; Tuncel *et al*., 2002) cancers in laboratory animals.

The type of cancer developed is dependent on the age, sex and strain of the laboratory animals, route of administration, concentration and the total dose of MNU, as well as the duration of the experimental period. Rats induced with MNU at young age (4 to 6-week-old) were reported to develop higher incidence of thymic lymphoma (Swenberg *et al*., 1975; Koestner *et al*., 1977; Mizoguchi *et al*., 1993) compared to middle-aged (52-week-old) and old age (98-week-old) rats (Mizoguchi *et al*., 1993). In particular, the middle-aged rats were found to likely to develop higher (97 - 100%) incidence of adenocarcinomas in the small intestine compared to young and old age rats (Mizoguchi *et al*., 1993). However, the administration of high dose of MNU (70 mg/kg body weight per injection for four injections) in young rats was found to have induced sub-acute haemolytic anaemia rather than lymphoma or leukaemia (Hazilawati *et al*., 2009).

The results of this study showed that all the rats treated with MNU (2 died during the experimental period) had enlargement of the lymph nodes, with 30% of the rats had hepatosplenomegaly and 7% had enlarged kidneys at necropsy. The organs were infiltrated with malignant lymphocytes in different grades, which were consistent with stage IV lymphoma, as described in the WHO's classification of human lymphoma (Jaffe *et al*., 2001). Meanwhile, normal architecture of those organs was diminished. Ironically, similar lesion was not observed in the thymus of all rats that had been treated with MNU. Using similar chemical, thymic lymphoma was observed in other studies (Koestner *et al*., 1977; Uwagawa *et al*., 1991; Hagiwara *et al*., 1993; Mizoguchi *et al*., 1993; da Silva Franchi *et al*., 2003).

It is important to note that the immunohistochemistry staining of the organs was positive for CD3 (*Fig. 1*), which is consistent with T-cell lymphoma. The results were comparable to those reported by Grompe *et al*. (1985), Kerja & Seidel (1986) and Marton *et al*.

(2008). However, they stated that most of the mice treated with MNU i.p developed thymic T-cell lymphoma as compared to peripheral T-cell lymphoma in this study.

The histopathological features of the T-cell lymphoma of spleen, lymph nodes, liver, lung, heart, and kidneys in this study were described as mild, moderate, and severe. In spleen, the malignant lymphocytes were generally characterised as undifferentiated, medium sized, bizarre pleomorphic nuclei with numerous mitotic figures in the red and white pulps (*Fig. 2*). The lesions were scored as mild as the malignant lymphocytes were individually present in the red pulp sinusoids and/or present as small focal aggregates in the splenic parenchyma. Normal architecture of the spleen was still preserved. Moderate lymphoma lesions were scored as the malignant lymphocytes surrounded the splenic cords in the red pulp and/or present as large focal aggregates in the spleen as well as in the periarterial lymphatic sheaths (PALS) which led to the enlargement of the white pulp. Other than those criteria, the presence of giant tumour cells and haemosiderin-laden macrophages in the red pulp are also important criteria. Severe lymphoma lesions were characterised by massive proliferation of malignant lymphocytes in the red and white pulps, leading to the loss of normal splenic architecture denoted by the absence of sinusoids and splenic cord in the red pulp, as well as marginal and follicular zones in the white pulp.

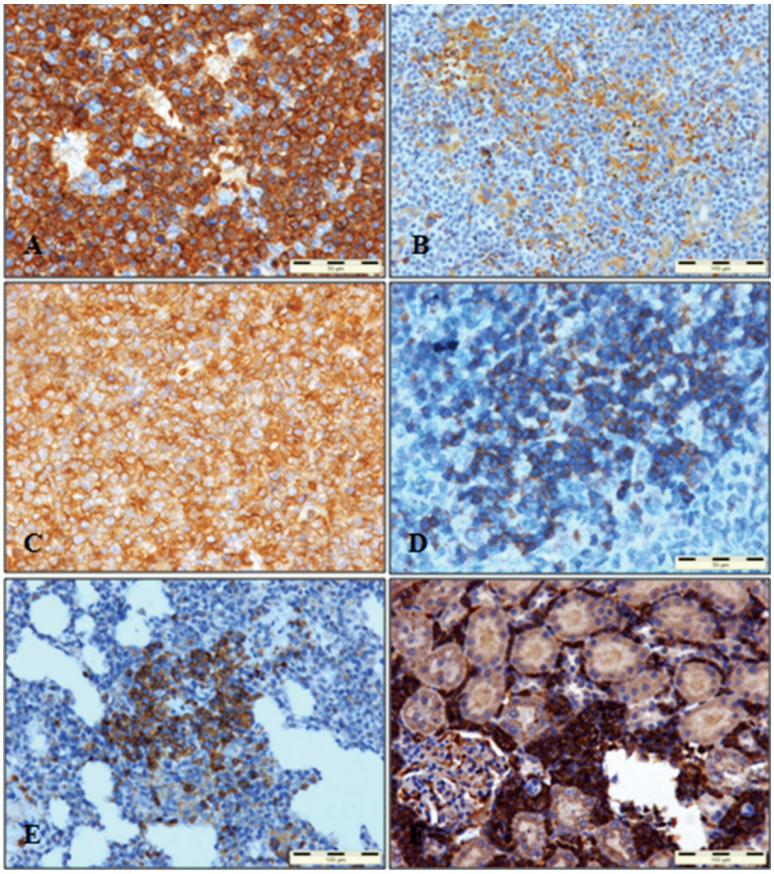

Fig. 1: Malignant T-lymphocytes in the lymph node (A), liver (B), spleen (C&D), lung (E) and kidney (F) stained positively for CD3 primary antibody

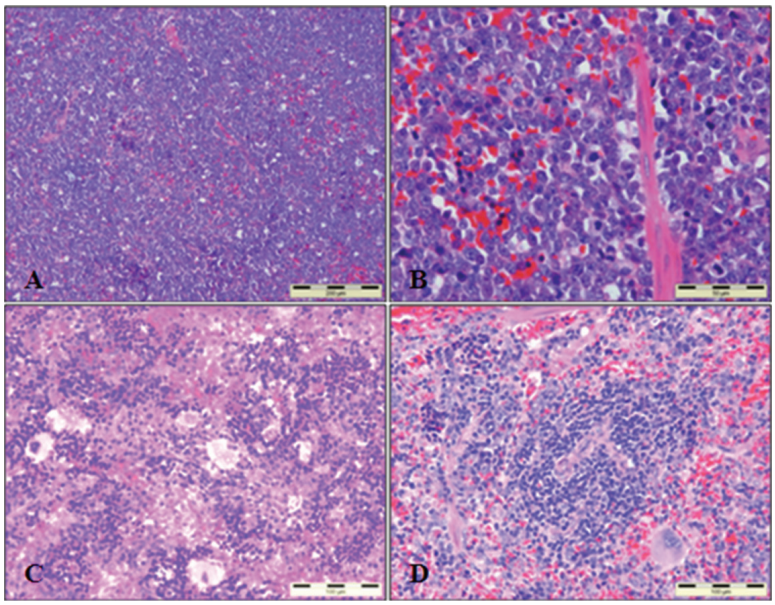

Fig. 2: Spleen; A&B) Normal architecture of the spleen, replaced by proliferation of numerous malignant lymphocytes (x10 & x40, respectively, H&E). C&D) Clusters of malignant lymphocytes in the red pulb (x20, H&E). Note one giant tumour cell in photomicrograph D and neoplastic lymphocytes surrounding the splenic cords in photomicrograph A to D

Generally, malignant lymphocytes in the lymph nodes, including auxiliary, mesenteric and submandibular, showed a similar criterion of malignancy as described in the spleen (*Fig. 3*). Meanwhile, mild lymphoma lesions were characterised as the presence of small focal aggregates of malignant lymphocytes in the paracortex and medullary areas of the lymph nodes. Infiltration of the malignant lymphocytes was occasionally observed in the subcapsular sinus. Moderate lymphoma lesions were described as the presence of large aggregates of malignant lymphocytes in the cortex, paracortex and medullary areas of the lymph nodes, leading to a partial loss of the normal lymph node architecture. Moderate infiltration of the malignant lymphocytes was observed in the subcapsular sinus. Severe lesions were denoted as the architecture of the cortex, paracortex, and medullary areas of the lymph nodes are diminished as a result of diffuse proliferation of malignant lymphocytes. The newly developed small blood vessels indicating angiogenesis were clearly observed in the lymph nodes. The subcapsular sinus was numerously proliferated by malignant lymphocytes and formed a neoplastic zone surrounding the lymph nodes.

Just like the spleen and lymph nodes, liver was infiltrated with malignant lymphocytes with a prominent criterion of malignancy. The size of the malignant lymphocytes varies from small to large. Hepatocytes were necrotised and congested (*Fig. 4*). Mild lesions were characterised as the presence of only a few malignant lymphocytes at the portal triads, necrotic hepatocytes with pyknotic nuclei and congested blood vessels. Moderate lesions were described as the presence of aggregates of malignant lymphocytes at the portal triads and also in the hepatic sinusoids, necrotic hepatocytes with karyorrhexis and karyolytic nuclei, and congestion of the blood vessels and hepatic sinusoids. Severe lesions were characterised as the presence of diffuse infiltration of malignant lymphocytes in the liver parenchyma. Hepatocytes were absent and/or only present as a small island, with 1 to

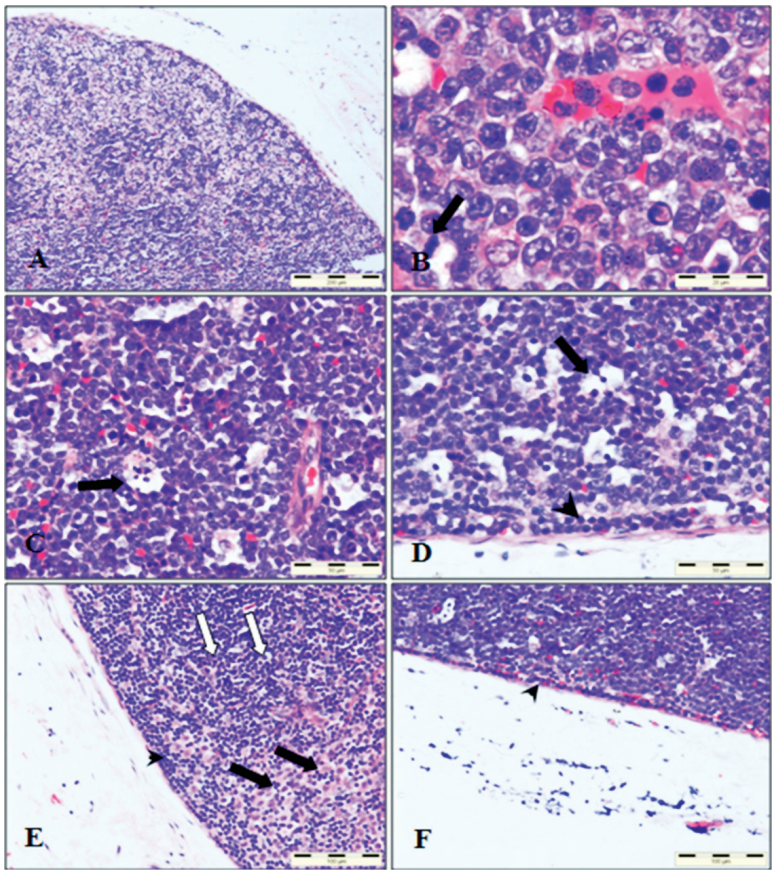

Fig. 3: Lymph node; A) Normal lymph node architecture is replaced by diffuse proliferation of small and large lymphocytes produces inconspicuous demarcation of structures in the cortex, which include trabeculae, lymphoid follicles and germinal centre, and medulla (x10, H&E). B) Numerous malignant lymphocytes characterised as marked cellular and nuclear pleomorphism (x100, H&E). Note that one mitotic figure (arrow). C & D) Numerous small blood vessels indicating angiogenesis (arrows) and infiltration of numerous malignant lymphocytes in the subcapsular sinus (arrow head) (x40, H&E). E & F) The subcapsular sinus (arrow heads) is infiltrated by malignant lymphocytes. E) Mixture of small (white arrows) and large lymphocytes (black arrows); nuclei of the large lymphocytes are paler and bigger indicating the cells are undergoing active proliferation (x20, H&E) (photomicrograph B, C, D, E, and F are magnification of photomicrograph A)

3 hepatocytes within the clusters of malignant lymphocytes. Meanwhile, liver parenchyma was severely congested.

Kidneys exhibited similar malignant lymphocyte characteristics as the spleen, lymph nodes and liver. The size of the malignant lymphocytes, however, varies from small to medium. The cells predominantly infiltrated into the interstitial space of the renal tubules, moderately infiltrated and surrounded the glomeruli. Numerous malignant lymphocytes were observed around the renal artery. Tubular epithelial necrosis was also observed (*Fig. 5*). Mild lymphoma lesions were characterised by the presence of metastatic neoplastic lymphocyte infiltration surrounding the large blood

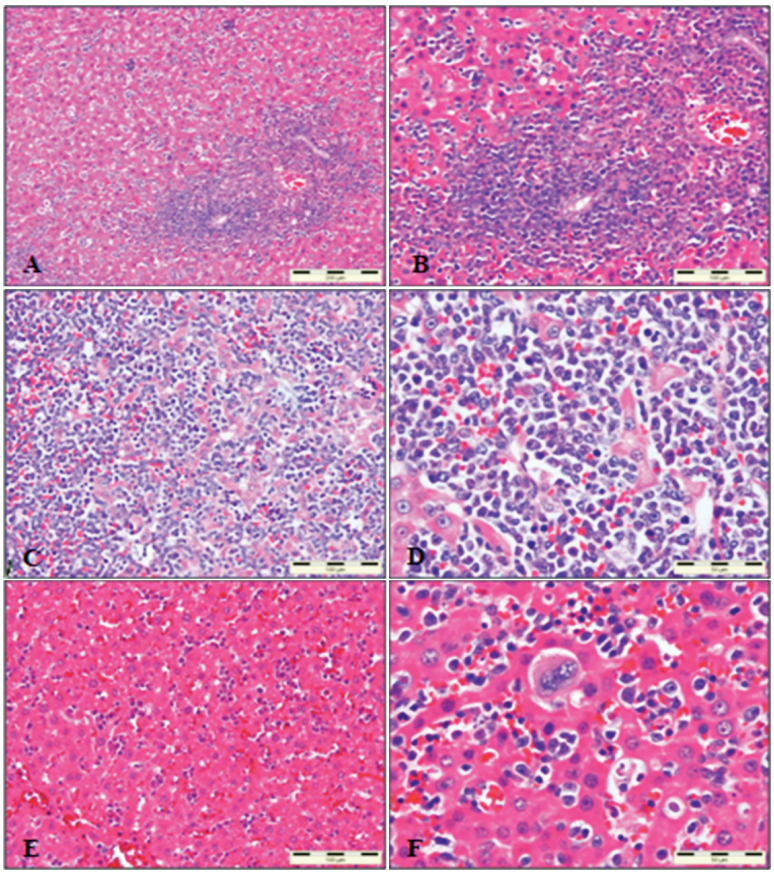

Fig. 4: Liver; A&B) Infiltration of numerous malignant lymphocytes at the portal triad area and in the hepatic sinusoids (x10 & x20, respectively, H&E). Note the congested blood vessel with malignant lymphocytes. C&D) Normal architecture of hepatocytes is replaced by diffuse infiltration of malignant lymphocytes (x20 & x40, respectively, H&E). Note that congested sinusoids and islands of hepatocyte surrounded by the malignant lymphocytes. E&F) Clusters of malignant lymphocytes in the hepatic sinusoids (x20 & x40, respectively, H&E). Note that one giant tumour cell in photomicrograph E (photomicrograph B, D and F are magnification of photomicrograph A, C and E, respectively)

vessels. For moderate lesions, similar lesions characterised for the mild lesions were observed, along with the presence of metastatic neoplastic lymphocyte infiltration in the renal interstitium of both cortex and medulla, as well as around the glomeruli. Congested blood present in the renal interstitium and large blood vessels. Epithelial necrosis of the renal tubules, which include the proximal, distal and loop of Henle, was occasionally observed. Severe lesions were described as the presence of predominantly medium sized metastatic neoplastic lymphocytes in the areas described for the mild and medium lesions, including the fatty tissue of the renal calyx/pelvis. The number of cells, however, was more abundant, which resulted in the reduction of the number of renal tubules. Congested blood in the large blood vessels contained numerous metastatic lymphocytes.

Lymphoma lesions in the lungs were exhibited by the infiltration of undifferentiated, small to medium size metastatic neoplastic

lymphocytes, with pleomorphic nuclei and mitotic activity, around the pulmonary blood vessels and as focal aggregates in the lung parenchyma. The clusters of neoplastic lymphocytes were also observed in the bronchus associated lymphoid tissue (BALT), leading to the enlargement of BALT (*Fig. 6*). Mild lymphoma lesions were characterised as the presence of a few number of small metastatic neoplastic lymphocytes around the large blood vessels. Moderate lymphoma lesions were observed by the presence of small to medium metastatic neoplastic lymphocytes around the pulmonary blood vessels and as small focal aggregates in the alveolar areas, resulting in the thickening of the alveolar walls. Meanwhile, enlargement of the BALT was observed as a result of the proliferation of the neoplastic lymphocytes. The cells were also observed to be present in the congested blood vessels and in the alveolar walls. For severe lesions, lung parenchyma was predominantly infiltrated by the neoplastic cells as large focal aggregates throughout the lung parenchyma, surrounding

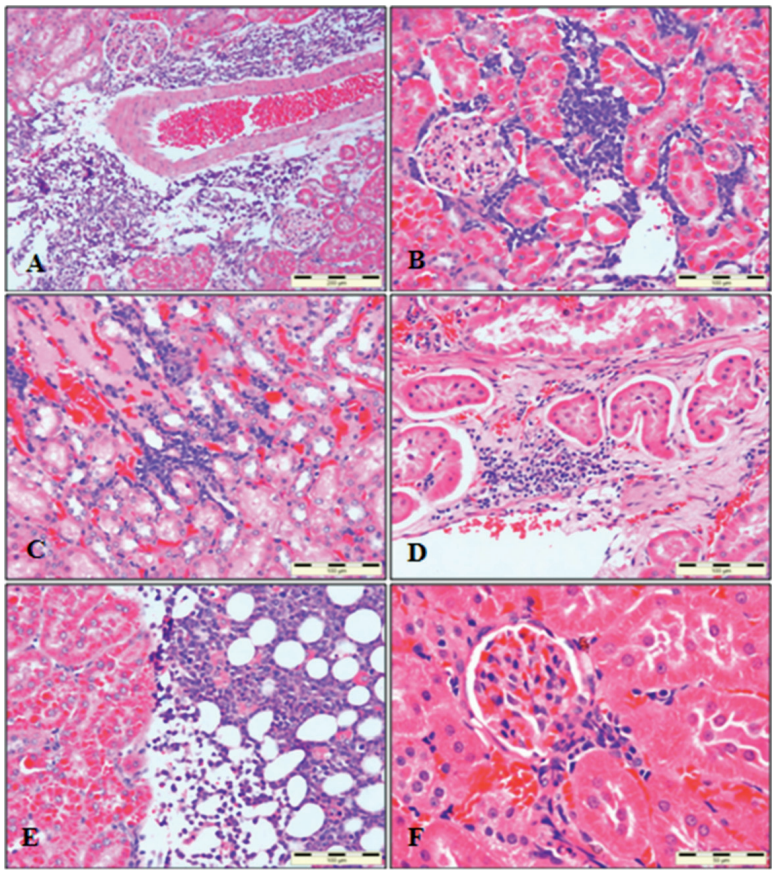

Fig. 5: Kidney; A) Infiltration of numerous malignant lymphocytes around the large blood vessel (x10, H&E). Note that the congested blood in the lumen of the renal artery contains malignant lymphocytes. (B, C & D) The malignant lymphocytes predominantly infiltrated the interstitial tissues of the renal tubules (x20, H&E). Note that the congested blood within the renal interstitial tissues in photomicrograph C. E) Infiltration of numerous malignant lymphocytes into the fatty tissues of the renal calyx/pelvis (x20, H&E). (B&F) Infiltration of the malignant lymphocytes surrounding/in the glomerulus (x40, H&E)

the small and large pulmonary blood vessels and also in the alveolar walls. The cells were proliferated in the BALT and this resulted in the enlargement of the BALT and the narrowing of the bronchial lumen. Congested blood with neoplastic lymphocytes and interstitial penuemonia were also noted.

The infiltration of metastatic neoplastic lymphocytes between the cardiac muscles was observed in the heart. Mild lesions were characterised as the presence of the neoplastic lymphocytes surrounding the coronary blood vessels. Moderate lesions were demonstrated as the presence of the neoplastic cells in between the cardiac muscles and also surrounding the blood vessels. Necrosis of the myocytes which led to loss of the normal myocyte coordination was also observed. Similar lesions were more obvious for severe lymphoma lesion in the heart, along with the aggregations of infiltrated neoplastic cells in the cardiac parenchyma (*Fig. 7*).

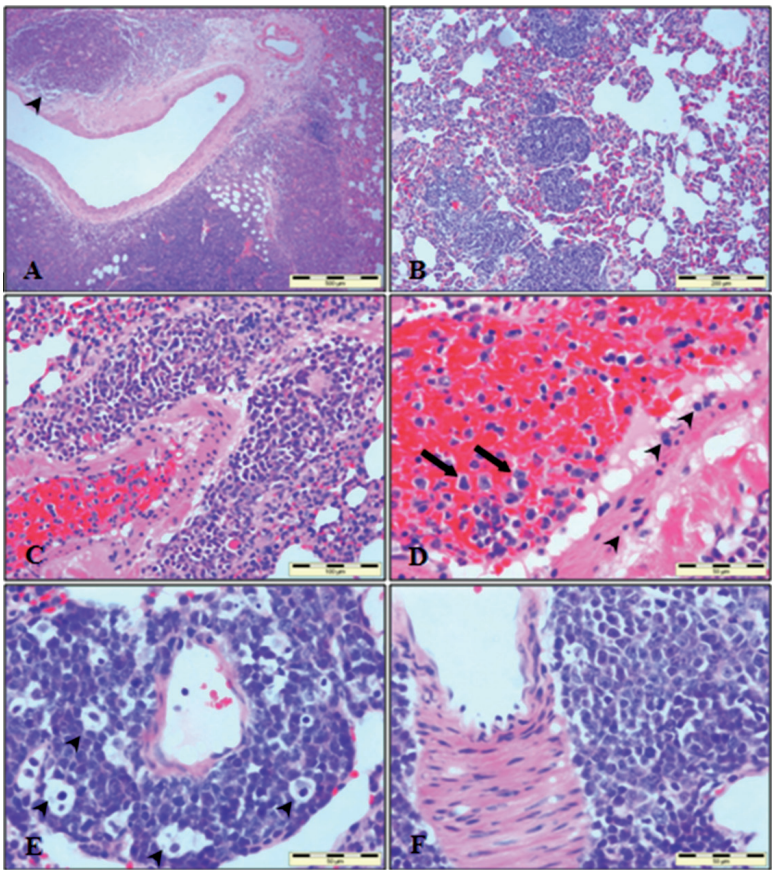

Fig. 6: Lung; A) Aggregation of malignant lymphocytes in BALT (arrowhead) (x4, H&E). B) Clusters of malignant lymphocytes infiltrated into the lung parenchyma (x10, H&E). C) Infiltration of numerous malignant lymphocytes around the pulmonary artery (x20, H&E). Note that presence of congested blood and malignant lymphocytes in the lumen of the pulmonary artery. D) Higher magnification of photomicrograph C clearly shows that the malignant lymphocytes are in the lumen (arrows) and wall (arrowheads) of the pulmonary artery (x40, H&E). E&F) The malignant lymphocytes are around the large and small blood vessels (arrowheads) (x40, H&E)

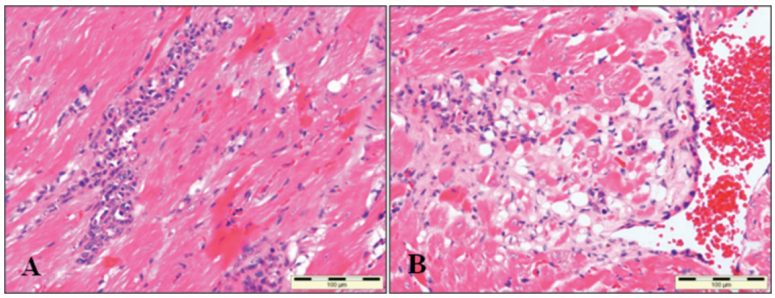

Fig. 7: Heart; A) Infiltration of malignant lymphocytes in between the cardiac muscles (x20, H&E). B) Fatty necrosis of the myocytes was observed around the large blood vessel which is infiltrated by the malignant lymphocytes (x20, H&E). Note that metastatic malignant lymphocytes line the intima of the large blood vessel. The congested large blood vessel contains a few malignant lymphocytes

CONCLUSION

The pathological features of stage IV peripheral T-cell lymphoma in rats, induced with MNU in different grades, have exclusively been described in this study. It is very useful for future study, particularly to evaluate the effectiveness or efficacy of new anti-tumor therapies.

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Urolithiasis in Boer Bucks

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ABSTRACT

This paper describes three cases of urolithiasis in adult Boer bucks. The affected bucks were among the 50 breeders kept under intensive system given cut and carry Napier grass at the rate of 2 kg/animal/day. In addition, the animals were also supplemented with commercial goat pellets at the rate of 300 g/animal/day, 200-300 g of palm kernel expeller (PKE) and mineral block. The affected animals showed clinical signs of stranguria, anorexia, prolonged urination, dribbling urine, tail flagging and abdominal pain. Prior to death, they appeared to be depressed, recumbent, and showed abdominal distension. Supportive treatments in the form of anti-inflammatory drugs and oral drench of ammonium chloride (1%) were attempted. All the animals in this study died within 2 weeks following the onset of depression. Post-mortem examinations revealed swollen testis and severe haemorrhages in the urethra with blackish sandy material deposited within the lumen. There were ascites and swollen kidneys, while cloudy and thick urine filled the bladder. Histological examinations revealed the presence of purplish sandy material, either within the lumen or closely associated with the wall of the urethra. The urethral areas where the calculi were in-contact with the wall showed extensive necrosis with destruction of the epithelial layer and haemorrhages. Urinary calculi have a complex aetiology, but management, nutritional, and anatomical considerations can be helpful.

Keywords: Urolithiasis, Boer bucks

INTRODUCTION

Urolithiasis is a condition of the urinary tract, in which insoluble mineral and salt aggregate around a nidus of proteinaceous material within the bladder or urethra (Belknap & Pugh, 2002). These stones consist of combinations of various minerals and come in many shapes and sizes. Once the stones become too numerous or too large, they cause obstruction of the urethra at the vermiform appendage, the ischial arch or the neck of the bladder (Pinsent & Cottom, 1987). Urolithiasis can rapidly progress to bladder or urethral rupture, uremic crisis, and death (Baxendell, 1984). Males are more likely to be affected as females generally have a shorter, wider urethra (Matthews, 1999). Uroliths can occur in all species but they are a common problem in domestic ruminants (Matthews, 1999; Belknap & Pugh, 2002).

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In the past, urolithiasis had rarely been reported in Malaysia. However, with the increase in the import of and goat rearing, especially the Boer goat, urolithiasis has been promoted in the recent years. This paper describes the occurrence and pathological changes in Boer goats with urolithiasis.

CASE DESCRIPTION

Case History

This report describes three cases of urolithiasis involving imported adult Boer bucks aged >2 years old. The affected bucks were among the 50 breeder males kept under the intensive system. They were fed cut and carry Napier grasses at the rate of 2 kg/animal/day and supplemented with commercial goat pellets at the rate of 300 g/animal/day, palm kernel expeller (PKE) and mineral block. The affected animals showed clinical signs of stranguria, anorexia, prolonged urination, dribbling urine, tail flagging, and abdominal pain. Prior to death, they appeared to be depressed, recumbent and showed abdominal distension. Supportive treatments in the form of anti-inflammatory drugs and oral drench of ammonium chloride (1%) were attempted. Nonetheless, all the affected animals died within 2 weeks following the onset of depression.

CASE FINDINGS

In all cases, the post-mortem examination revealed swollen testis due the accumulation of fluid in the scrotum (*Fig. 1*). The prepuce was stained while the abdomen was distended, containing fluid (*Fig. 2*). Upon opening the urogenital system, there were severe haemorrhages and necrosis along the urethra with blackish sandy material within the lumen (*Fig. 3*). The urinary bladder contained cloudy and thick urine, while the kidneys were swollen with hydronephrosis (*Fig. 4*).

Histopathological examination revealed the presence of purplish sandy material, either within the lumen or closely associated with the wall of the urethra (*Fig. 5*). The urethral areas, where the calculi were in-contact with the wall, showed extensive necrosis with destruction of the epithelial layer and haemorrhages with numerous inflammatory cells (*Fig. 6*).

Fig. 1: Swollen testis with subcutaneous oedema and soiled prepuce

Fig. 2: Fluid-filled the abdominal cavity

Fig. 3: Urethra of affected goat showing necrosis and accumulation of blackish, sandy materials

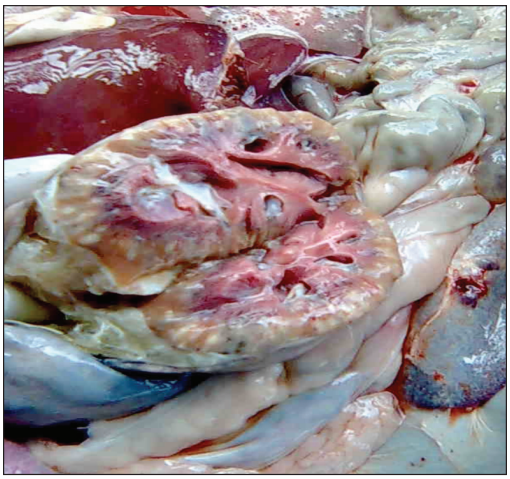

Fig. 4: Swollen kidneys with evidence of hydronephrosis

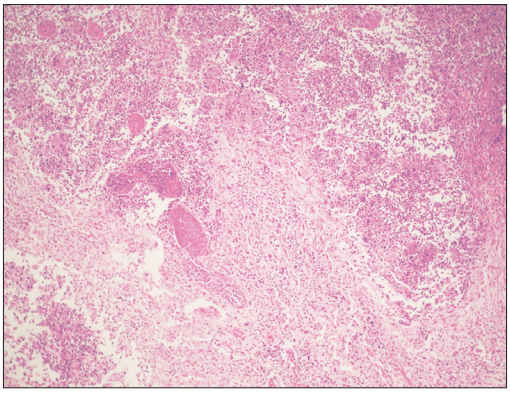

Fig. 5: A photomicrograph of an affected urethra showing the presence of urolith and surrounding necrosis (HE x40)

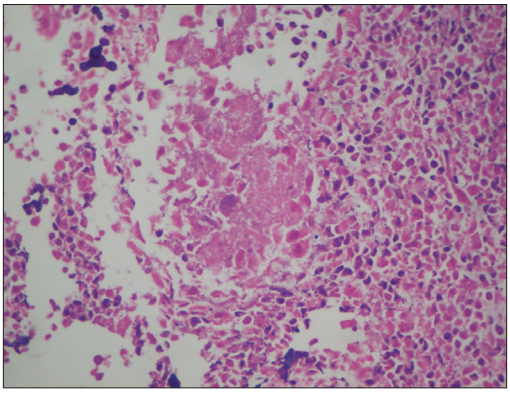

Fig. 6: A photomicrograph showing the urolith, haemorrhages and inflammatory cells (HEx400)

DISCUSSION

Goats of various breeds and purposes have been documented with urolithic stone problems in captivity (Bellenger *et al*., 1981; Smith & Sherman, 1994; Gutierrez *et al*., 2000). These stones often occur when concentrated or supplemented feed with high phosphorus content is presented to the goats (Blood *et al*., 1989; George *et al*., 2007). Therefore, diet and animal management are considered as important risk factors in the formation of uroliths in ruminants (Blood *et al*., 1989; Kahn *et al*., 2005; George *et al*., 2007). Although the relationship between diet and urolith formation in goats has not been clearly established, previous studies have suggested that particular diets contribute to the formation of struvite calculi (Blood *et al*., 1989; Aitken, 2007). High-concentrated

grain diets with approximately equal proportions of calcium and phosphorus and diets high in magnesium, potassium, and phosphorus are thought to predispose animals to calculi (Huang *et al*., 1999).

Therefore, obstructive urolithiasis remains an often deadly problem for male goats, kept intensive. The solution involves understanding and implementing a proper balance of minerals and nutrients in their diets, while mitigating environmental factors to minimize the risk of this disease.

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Pulmonary Lesions Associated with Intratracheal Benzo(a)pyrene Instillation in Sprague Dawley Rats

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ABSTRACT

This study was conducted to assess acute exposure to very low dose of intratracheally instilled Benzo(a)pyrene (BaP) on the lungs of rats. A total of 30 rats were utilized in this study and they were randomly divided into 6 groups. The control group (G1) did not receive any treatment, whereas the rats in the remaining 5 groups were administered with 13.8 ng of BaP, which were then sacrificed at 1 hour (G2), 8 hours (G3), 16 hours (G4), 32 hours (G5), and 72 hours (G6) of post-instillation (p.i.). Morphological appearances of all the lungs of all the treated rats consisted of various degrees of congestion, mostly evident in G3 and early development of emphysema, as seen in G4. These worsened as time progressed as observed in G6. On the other hand, the histological findings of the lungs of the treated rats revealed that the lungs had underwent some changes that were characterized by progressive alveolar congestion, epithelialisation with emphysema and accompanied by infiltration of inflammatory cells predominantly with alveolar macrophages and some neutrophils. However, even with such lesions seen, there was no apparent manifestation of impairment of the pulmonary system.

Keywords: BaP, histological, intratracheally, lung, morphological

INTRODUCTION

Air pollution has been incriminated countless times as the cause of the adverse health effects reported in man (Martin *et al*., 1997; Chauhan & Johnston, 2003). There are evidences of increased in respiratory related illnesses which have been linked to the occurrences of air pollution (Kelly, 2003; Risom *et al*., 2005). Malaysians have suffered too many episodes of haze in the last few decades, with one of the worst in 1997, where Benzo(a)pyrene (BaP), i.e. a form of polycyclic aromatic hydrocarbon (PAH) and categorized as a particulate matter (PM), was found to be the most predominant pollutant (Zakaria *et al*., 1998). This BaP, which is notoriously known to possess both carcinogenic and mutagenic properties, is usually found as a product of incomplete combustion (ATSDR, 1995; Faust, 1994) ubiquitously in the environment and has extensively been used as an indicator of carcinogenic hazard in air pollution studies (Khesina, 1994). Even though many studies have been conducted using animal models and epidemiological data exist linking the presence of the hazardous BaP to cases of pulmonary insufficiency (Brauer *et al*., 2001), the exact mechanism behind the cause and effect is still poorly understood (Lin *et al*., 2009). There is also limited information on exposure effects to the pollutant via inhalation at such a very low dose at certain duration (ATSDR,

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1995), such as the one that was experienced by the public during the severe haze episode in 1997 in Malaysia.

MATERIALS AND METHODS

Thirty, 8-9 weeks old, male, Sprague Dawley rats weighing between 150-200g, were used as models in this study. Rats were housed in separate cages, whereby 3 rats were place per cage. All the rats had complete access to standard rat chowder and water *ad libitum*. All the rats were allowed a week of acclimatization prior to the initiation of the treatment. As precaution against any bacterial infections, a single long acting oxytetracycline injection (10 mg/kg) was intramuscularly administered to all rats.

The rats were equally and randomly assigned to 6 different treatment groups, encompassing the control (G1), 1 hour p.i. (G2), 8 hours p.i. (G3), 16 hours p.i. (G4), 32 hours p.i. (G5), and 72 hours p.i. (G6), whereby the rats were humanely sacrificed at the respective intervals. The rats in G1 were only sacrificed at 72 hours.

A total of 7 µl of BaP in tricaprylin mixture, which is equivalent to 13.8 ng of BaP, was intratracheally instilled once, as described by Oka *et al*. (2006) under general anaesthesia to each rat in the treatment group, while the control rats did not receive any instillation. The dose was calculated based on the concentration of BaP during the 1997 haze in Malaysia which lasted for 3 months (*Zakaria et al*., 1998).

The rats were closely monitored for any development of clinical signs pertaining to the respiratory system dysfunction. After each interval had been reached, the rats were put under general anaesthesia using a combination of ketamine hydrochloride (60 mg/kg) and xylazine hydrochloride (10 mg/kg) that were intramuscularly given and later humanely euthanized. The lungs were immediately procured and visual appraisal of the changes was noted before the lungs were fixed in 10% neutral buffered formalin for histopathological evaluations using Haematoxylin and Eosin (H&E) staining.

RESULTS AND DISCUSSION

No obvious clinical sign was observed in any of the rats. This could have been either due to the very low dose used or because the lung has a very large reserve whereby only when more than 75% of the reserve is exhausted, manifestations of the clinical sign can be noted. The expected clinical signs would generally include depression, inappetance and inactiveness, whilst signs of respiratory impairment comprised of tachypnoea, hyperventilation, dyspnoea, and in worst cases, cyanosis. However, it is believed that common air pollutants which often cause injuries to Type I alveolar cells can lead to mild to moderate permanent lung lesions. Even though large accumulations of inflammatory cells were seen in the lung which had previously been exposed to pollutants, it was subsequently insufficient to produce apparent lung lesions (Witschi, 1990) and thus, could probably explain the absence of any obvious clinical signs.

Collectively, there were a few clear gross pathological changes seen in the lungs of all the rats that were treated with BaP. The lesions include varying degrees of congestion, which were mostly distinguished in G3 rats (4/5), with emphysema observed earliest in G4 rats (5/5). However, these lesions worsened and became most apparent in G6 rats (5/5) (*Fig. 1*). On the other hand, this pulmonary congestion was not observed in any of the control rats.

Based on the findings of this study, it is clear that acute exposure to even a minute dose of BaP induced lung injuries, as can be evident from the presence of inflammatory cells, particularly the alveolar macrophage and neutrophil infiltrations in the alveolar spaces, accompanied by alveolar congestion, alveolar epithelisation, metaplasia, and dysplasia (*Fig. 2*), and followed by the early development of emphysema, as characterised by the 'thinning' appearance of the alveolar septa due to the outstretching with subsequently rupture of the alveolar wall (*Fig. 3*).

In a study done by Emre *et al*. (2007) in rats given 200 mg/kg of BaP intraperitoneally and sacrificed 24 hours later, the histological findings in the lungs showed marked thickness of alveolar septae, inflammatory cells infiltration, and

cellular debris in the lumen of the bronchioles. There was also alveolar space enlargement, which was accompanied by thinning as well as destruction of the septal wall. Similar changes in this experiment were particularly observed at 32 hr p.i with BaP (G5) (4/5).

Venugopal *et al*. (2007) reported that mice sacrificed 18 weeks after an oral treatment with BaP (50 mg/kg) were found to have developed severe alveolar changes, such as increased number of hyperchromatic, and irregular nuclei in the cells of the alveolar walls. Meanwhile, hamsters that were given BaP developed pleomorphic nucleoli and squamous metaplasia in their lungs (Smith *et al*., 1975). All the reported observations are generally similar to the findings in this study, as shown in *Fig. 2*. Das *et al*. (2007) also observed that dysplasia was very prominent in the chronically BaP (0.2 mg/mouse) exposed mice 8 weeks post-exposure and onwards. Cellular dysplasia strongly indicates an early sign of cancer and is typified by the 4 major pathologic microscopic changes, namely anisocytosis (cells of unequal size), poikilocytosis (cells with abnormal shape), hyperchromatism and the presence of mitotic figure, as seen in *Fig. 2*.

Emphysema, as observed in this study, is also reported by others as a *sequalae* of smoking (e.g. Hautamaki *et al*., 1997; Li *et al*., 2003).

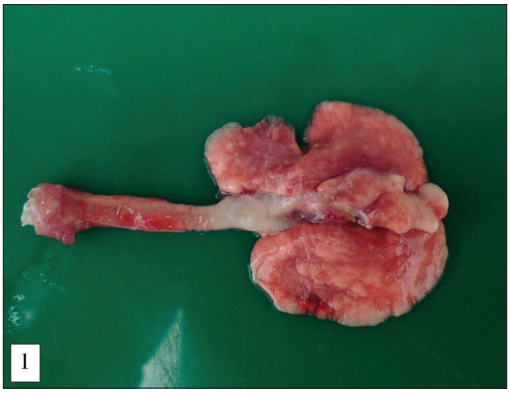

Fig. 1: Photograph of a rat's lung at 72 hours p.i. with BaP (G6) showing mild to moderately congested lung with very prominent emphysema at the lung lobes

Cigarette smoking also exposes an individual to the harmful effects of BaP. The findings of this study are almost similar to one by Li *et al*. (2003) who exposed rats to cigarette smoke. Rats developed inflammation in the lungs, as characterized by alveolar septal thickening, presence of RBCs and chronic inflammatory cells, mainly macrophages which were scattered within the alveolar spaces accompanied by diffused areas of emphysema involving the whole lung besides the finding of cellular debris in the bronchioles and the thickening of the blood vessel walls, and with a reduction in the size of the lumen. In this study, all the lung lobes, particularly the peripheral lung regions, were severely emphysematous and the inflammatory cells were found throughout the lungs.

The possible pathomechanism behind the occurrence of emphysema was believed to have been due to the alveolar macrophages releasing many proteolytic enzymes where the macrophages were accounted for more than 90% of the inflammatory cells in the smoker's

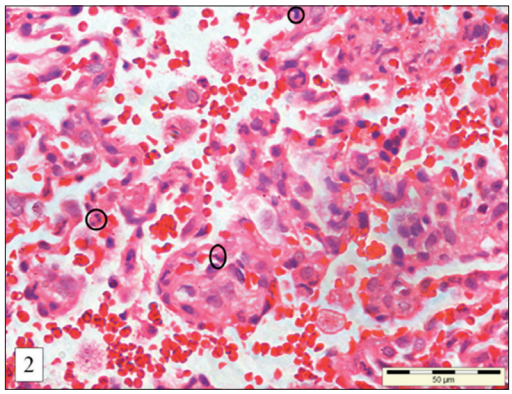

Fig. 2: A photomicrograph of the histological section of the lung of a BaP treated rat at 8 hours p.i. (G3) depicting a lesser dense area of the lung with severe inflammatory reactions predominantly by alveolar macrophages and mild neutrophils infiltration, and accompanied by severe haemorrhages in alveolar spaces and thickened alveoli. Some of the cells were also hyperchromatic and had irregular nuclei. There is also a presence of degenerated and necrotic cells. Mitotic figure was also visibly seen (indicated by black circles) [H&E; x400]

lungs, as evident from this study. This is on the contrary to the belief that only neutrophils are responsible in secreting elastase which results in the breakdown of elastic fibres. Macrophages can release a specific enzyme, known as macrophage elastase, i.e. a metalloproteinase that can solubilise various extracellular matrix proteins and elastin. This enzyme is also believed to be responsible in generating monocytes chemotactic activity which causes recruitment of more monocytes in the lungs. In addition, this enzyme can also make α_1-antitrypsin inactive, which will indirectly enhance the elastase activity of neutrophils (Hautamaki *et al*., 1997; Lucattelli *et al*., 2003).

The elastase released by leukocytes encompasses both the alveolar macrophages and neutrophils which can be cytotoxic to the endothelial cells and may subsequently lead to increase vascular permeability or vasculitis (Lee & Downey, 2001). In view of the fact that blood vessel wall is made of elastic and collagen fibres; this possibly makes it vulnerable to be digested by elastase. Thus, there could very well be change in the permeability or necrosis leading to the escape of erythrocytes into the alveolar spaces, as illustrated in *Fig. 3*. Prolonged secretion of elastase and other neutral proteinases can lead to breakdown of elastic tissues and damage to blood vessels (Werb & Gordon, 1975).

Recently, there has been much interest in the theory of oxidative stress caused by air pollutants, resulting in the degeneration and necrosis of cells. This is due to the disruption in the integrity of a cell from the attack of free radicals on the very vulnerable phospholipid layers (Kelly, 2003; Kooter, 2004). Recent evidences suggest that BaP may be able to induce oxidative stress conditions in living systems and therefore causes oxidative damage to macromolecules such as protein, lipid, and DNA (Garcon *et al*., 2001).

The findings of the cellular debris in the lumen of the bronchioles (see *Fig. 4*) can be explained as the result of degeneration as well as necrosis of the epithelial cells lining the bronchioles leading to the sloughing of dead cells into the lumen. Since BaP was introduced intratracheally, the bronchioles have direct contact with the pollutant and in view of the fact that the cell membrane (which consists

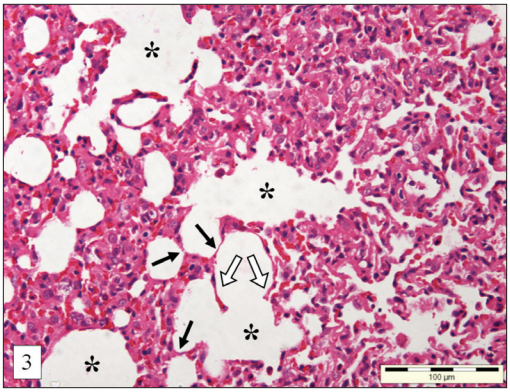

Fig. 3: A photomicrograph of the histological section of the lung of a rat treated with BaP at 32 hours p.i. (G5) at a less dense area near the lung periphery, exhibiting a moderate alveolar congestion, clusters of alveolar macrophages, stretching of alveolar wall (arrow) and rupture of alveoli (arrowheads) with emphysema (asterisks. [H&E; x200]

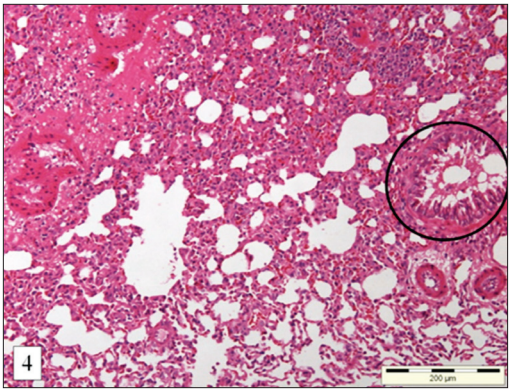

Fig. 4: A photomicrograph of the histological section of the lung of a BaP treated rat at 72 hours p.i. (G6), exhibiting alveolar hyperplasia at the top, whereas emphysema was seen to have distributed all over the lung mainly at the bottom area. Notice the cellular debris in the bronchiole (marked by black circles) [H&E; x100]

of phopholipid layers) is very susceptible to the attack of free radicals generated by BaP, it could have resulted in bronchiolitis. Werb & Gordon (1975) commonly observed recurrent bronchiolitis as a primary response to dusts and air pollutants.

The results of this study also demonstrated that BaP might induce an intense influx of immune cells in the lungs at around 8 to 16 hours p.i with BaP. Emre *et al*. (2007), Kooter (2004) and Kelly (2003) stated that immune cells influx is a prominent feature in response to exposure to PM. The blood-air barrier migration behaviour by the inflammatory cells might have triggered a proliferative response in the alveolar epithelium (Witschi, 1990).

The most vulnerable cell to toxic agents, particularly from air pollutants in the lung, is Type I alveolar cell (Kooter, 2004; Kelly, 2003; Rahman & MacNee, 2000). Owing to its odd shape and localization in the alveolar epithelium which precludes it from undergoing cellular division to replace damaged cells. Thus, the damaged cells are slowly being replaced by Type II alveolar cells that have taken up the shape and functional role after undergoing the division process.

It is believed that common air pollutants often cause injuries to Type I alveolar cells and will usually leave only mild to moderate permanent lung lesions (Witschi, 1990). This could have resulted in the architectural changes and cell proliferation, as well as cellular changes seen from the histopathological findings of lungs of rats which had received the BaP treatment in this study. The transition of Type I cells to form Type II alveolar cells is a strong indication that the lung is responsive and adapting to the assaults by BaP.

CONCLUSION

In conclusion, it has been shown that acute exposures of the lung to BaP can illicit damaging pulmonary changes, even at very minute dose. This is apparent from the morphological changes which include congestion of the lungs with a subsequent development of emphysema, whereas histological alterations were characterized by the progressive alveolar congestion, epithelialisation with emphysema, and accompanied by the influx of inflammatory cells, mainly by alveolar macrophages and some neutrophils. However, these lesions occurred without the manifestation of clinical signs pertaining to pulmonary system dysfunction. Nevertheless, more studies need to be done to establish the possibility of the involvement of oxidative stress caused by BaP in disrupting cellular viability which could have led to the occurrence of degeneration and necrosis of exposed cells.

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Detection of Bcl-2 Gene in Leukaemic Rats Using an EvaGreen Real-time RT-PCR Assay

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ABSTRACT

Bcl-2 is an anti-apoptotic gene that is involved in the apoptosis process. Suppression of apoptosis by anti-apoptotic gene can contribute to the occurrence of diseases such as leukaemia. The objectives of this study were 2-folds: first, to compare the sensitivity of an EvaGreen quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) with a conventional RT-PCR for the amplification of the Bcl-2 gene; second, to determine the expression of the Bcl-2 gene in *N*-methyl-*N*-nitrosourea (MNU)-induced leukaemia in rats using the EvaGreen qRT-PCR assay. A total of 32 male Sprague Dawley rats were assigned into two groups (n=16), namely, control and MNU groups. In particular, MNU was administered intraperitoneally (i.p) at a dose of 60 mg/kg body weight per injection at two times per week for 2 consecutive weeks. The rats were sacrificed after five months and blood samples were collected for RNA extraction and haemogram. The RNAs were converted into cDNA and amplified using both the EvaGreen qPCR and the conventional PCR assays. All the results were normalised with a housekeeper gene, i.e. glyceraldehyde 3-phosphate dehydrogenase (GADPH). The products of amplification were run on gel electrophoresis and all the results were then compared. Based on the relative intensity of the bands, the EvaGreen qRT-PCR assay was highly sensitive compared to the conventional RT-PCR assay as the Bcl-2 gene could not be amplified using the conventional RT-PCR. Interestingly, the results in this study showed that the expression of Bcl-2 was higher in rats with marked lymphocytosis as compared to the leukaemic rats with normal to mildly increase in lymphocyte count. In conclusion, EvaGreen qRT-PCR assay is more sensitive compared to the conventional RT-PCR, and Bcl-2 gene is abundantly expressed in leukaemic rats with marked lymphocytosis compared to the leukaemic rats with normal to mildly increase in lymphocyte number.

Keywords: Bcl-2, leukaemia, *N*-methyl-*N*-nitrosourea (MNU), EvaGreen qRT-PCR, conventional RT-PCR assays

INTRODUCTION

Apoptosis or programmed cell death is defined as a mechanism of cellular suicide which occurs after sufficient cellular damage. Apoptosis differs from necrosis and it is involved in the development of many diseases, such as cancer (Arends & Wyllie, 1991; Nikitakis *et al*., 2004; Loro *et al*., 2003; 2005). Various imbalances in the apoptotic system, such as insufficient

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amount of apoptosis, can lead to the development of autoimmunity and lymphoma by failing the process of lymphocyte death (Maniati *et al*., 2008). Apoptosis genes are thought to be useful as cancer biomarkers as they mark the expression of genes that control the apoptosis process. A group of proteins called B-cell lymphoma 2 (Bcl-2) family control the apoptosis process in cells (Goodsell, 2002). In general, Bcl-2 family proteins can be divided into proapoptotic and anti-apoptotic genes. Meanwhile, Bcl-2 functions as anti-apoptotic gene as it exhibits the ability to inhibit the apoptosis process and known as the most important gene of the Bcl-2 family (Reed, 1994; Lu *et al*., 1996). The Bcl-2 gene encodes a membrane protein localised in the nuclear membrane, the inner surface of mitochondria, and the endoplasmic reticulum (Armstrong, 2006), and it was first discovered by analysis of the t(14; 18) chromosomal translocation associated with human follicular B-cell lymphoma (Tsujimoto *et al*., 1985; Cleary *et al*., 1986).

In this study, the induction of leukaemia in male Sprague Dawley rats was performed by injection of *N*-methyl-*N*-nitrosourea (MNU). *N*-methyl-*N*-nitrosourea (MNU) was found to have induced tumours in many parts of the studied animals (Koestner *et al*., 1977; Kunze *et al*., 1989; Maekawa *et al*., 1985; Ogiu *et al*., 1977; Tsuda *et al*., 1983; Bosland & Prinsen, 1990; Hazilawati *et al*., 2010; Hutheyfa *et al*., 2011), as it is a very powerful carcinogen. In addition, it is also an alkylating agent, and exhibits its toxicity by transferring its methyl group to nucleobases in nucleic acids. Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) was chosen as a method of analysis for the transcription of Bcl-2 in this study. This technique has become an established procedure for quantifying the levels of gene expression, as well as gene rearrangements, amplifications, deletions or point mutations (Ponchel, 2007). Some examples of the chemistries available for qPCR are SYBR Green I and EvaGreen dyes. They are fluorogenic dyes that exhibit little fluorescence when in solution, but emit a strong fluorescent signal upon binding to double-stranded DNA.

The aims of the present study were to compare the sensitivity of the EvaGreen qRT-PCR assay with the conventional RT-PCR, and to determine the expression of Bcl-2 gene in the MNU-induced leukaemic rats using the method developed above.

MATERIALS AND METHODS

Animals and Experimental Design

A total of 32, 6-week-old, male Sprague Dawley rats were purchased from the Laboratory Animal Resource Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia (UKM) in Bangi, Selangor and placed in an individual polycarbonate rat cage in a colony animal room maintained at 22-27°C, with 40-70% humidity, and 12-hour light/12-hour dark conditions. The animals were acclimatised for two weeks and given commercial rat pellets and water *ad libitum* prior to the experiment. The rats were divided into 2 groups (n=16), namely control and MNU group. The rats in the control group were injected with normal saline intraperitoneally (i.p), whereas those in the MNU group were injected with *N*-methyl-*N*-nitrosourea (MNU) (Sigma-Aldrich, USA) dissolved freshly in normal saline, i.p, twice a week for 2 consecutive weeks at a dose of 60 mg/kg/body weight per injection (with a total dose of 240 mg/kg body weight). The animals were humanely sacrificed by bleeding under anaesthesia after five months. Their blood samples were collected from the heart via cardiac puncture for RNA extraction. These blood samples were also collected and examined for the evidence of leukaemia by Hutheyfa *et al*. (2009).

RNA Extraction

Total cellular RNAs were extracted using a QIAamp RNA Blood Mini Kit (Qiagen, USA) according to the manufacturer's instruction, from fresh blood collected in heparinised tubes. The optical density (OD) of the extracted RNA

was determined by measuring the absorbance at A260/A280 nm using a spectrophotometer (Eppendorf, German) and kept in -80°C until reverse transcription for cDNA synthesis.

Amplification of Bcl-2 gene Using a qRT-PCR Assay

The extracted RNA was converted into cDNA using a GeneAmp RNA PCR Core Kit (Applied Biosystems, USA) as per manufacturer's instruction. Master mix for qPCR was prepared with total reaction mixture of 20 uL contained 10 uL ImmoMix™ (Bioline, USA), 1 uL EvaGreen™ Dye (Biotium, USA), 1.5 um Bcl-2 forward and reverse primers (1st BASE, Malaysia), and 2uL of the sample containing 3 - 4 ng of cDNA mixed with sterile deionised water in a single real-time PCR tube for each reaction. The conditions for Bcl-2 amplifications were as follows: 1 cycle of 95°C for 10 minutes (predenaturation), followed by 37 cycles of 94°C for 20 seconds (denaturation), 64°C for 30 seconds (annealing), and 72°C for 45 seconds (extension). The amplification was done using a real-time PCR machine (Bio-Rad CFX96).

The following Bcl-2 primer sequences specific for rats, which were previously described by Brambrink *et al*. (2000), were used: Bcl-2 antisense, 5'-TTTCATATTT GTTTGGGGCAGGTC-3' and Bcl-2 sense, 5'-ATGGGGTGAACTGGGGGAGGATTG-3'. The size of PCR product is 350 bp.

Amplification of Bcl-2 and GAPDH Genes Using Conventional RT-PCR Assay

The same cDNA samples converted from the extracted RNA were used. Master mix for the conventional PCR was prepared using sterile deionised water with total reaction mixture consisted of 20 uL containing 2 mM $MgCl_2$ solution, 1× PCR Buffer II, 200 uM for each dNTPs (Applied Biosystems, USA), 1.5 uM Bcl-2 or GAPDH forward and reverse primers (1st BASE, Malaysia), 0.05 U AmpliTaq DNA polymerase (Applied Biosystem, USA) and 2 uL of sample containing 3 - 4 ng of cDNA. The amplification conditions for Bcl-2 gene were as follows: 1 cycle of 95°C for 2 minutes (predenaturation), followed by 37 cycles of 95°C for 30 seconds (denaturation), 61.4°C for 30 seconds (annealing), 72°C for 30 seconds (extension), and 1 cycle at 72°C for 7 minutes for the final extension. Similar amplification conditions were used for the GAPDH gene with 29 cycles and an annealing temperature of 55°C. The amplification of the cDNA was performed using a conventional PCR machine (Little Genius, BIOER).

Similar Bcl-2 primers used in the qRT-PCR were used for the conventional RT-PCR. The following primer sequences were used for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene; GAPDH sense: 5'-TGTGAACGGATTTGGCCGTA-3' and GAPDH antisense: 5'-CATTTGA TGTTAGCGGGATC-3' (GenBank accession number of the sequence is NM 017008) with the PCR product of 240 bp (designed using the Oligo Primer Analysis Software v.7).

Gel Electrophoresis

Ten microlitre of Bcl-2 qRT-PCR, Bcl-2 conventional RT-PCR and GAPDH conventional RT-PCR products were electrophoresed at 80V for 60 minutes in 1.5% agarose gel, stained with 0.5 ug/mL ethidium bromide solutions for 15-20 minutes and visualised under UV transillumination (Alpha Imager, GEL DOC).

RESULTS AND DISCUSSION

Several studies have shown that qRT-PCR is highly sensitive compared to conventional RT-PCR (Di Trani *et al*., 2006; Gurukumar *et al*., 2009). In spite of its sensitivity, the results produced by qRT-PCR are more rapid as gel electrophoresis is no longer necessary. However, qRT-PCR can be highly expensive, depending on the types of dye used. Hence, attempt was made to develop and optimise conventional RT-PCR, which is less expensive. The results of this study showed Bcl-2 was unable to amplify using the conventional RT-PCR assay for both the control

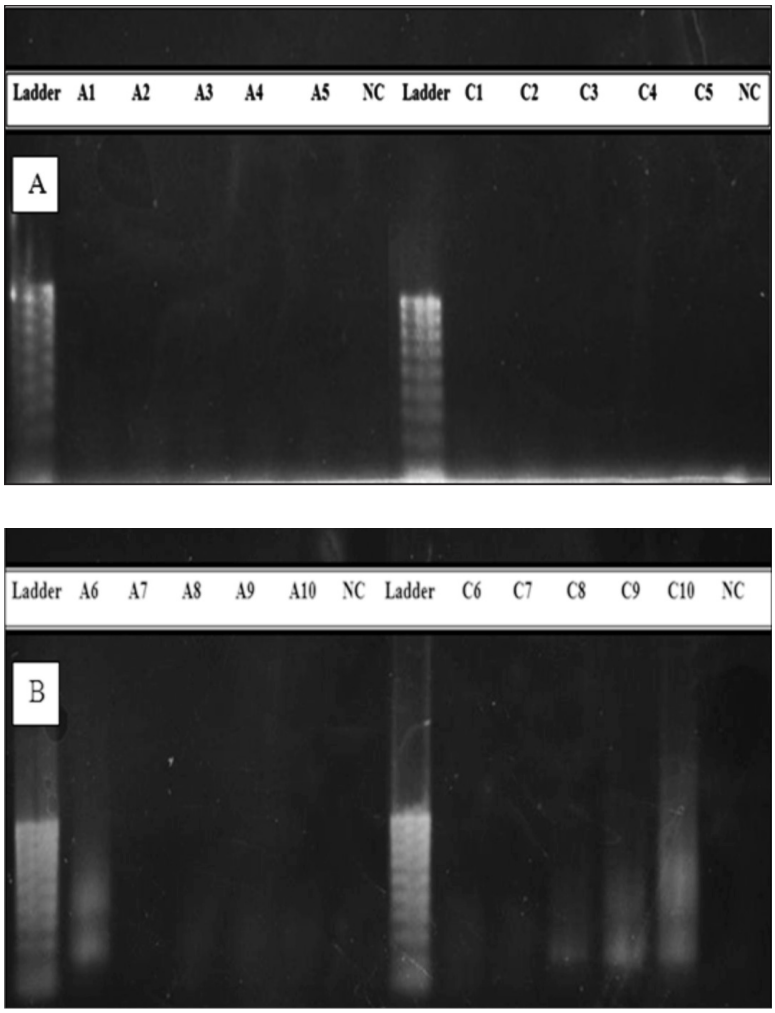

Fig. 1A and B: Bcl-2 gene amplification products of control (A1, A2, A3, A4, A5, A6, A7, A8, A9, and A10) and leukaemic (C1, C2, C3, C4, C5, C6, C7, C8, C9, and C10) rats amplified using conventional RT-PCR. 100 bp DNA markers (Bioline, UK) are shown in lanes 1 and 8 for each picture. Negative controls (NC) show no amplification. Note that no bands were amplified in A, whereas a few unspecific PCR products were amplified in B

and leukaemic rats (*Fig. 1A* and *1B*). The results were normalised by further analysis of the cDNA of the control and leukaemic rats using GAPDH primer via conventional RT-PCR, whereby the total RNA was clearly shown as succesfully extracted (*Fig. 2A* and *2B*).

Meanwhile, haematological results revealed that all rats induced with MNU had leukaemia with 30% and 70% of the rats had marked lymphocytosis and normal to mildly increase in lymphocyte count, respectively (Hutheyfa *et al*., 2009). The expression of Bcl-2 gene of the leukaemic rats was determined via gel electrophoresis of the EvaGreen qRT-PCR products (*Fig. 3*). The finding showed that leukaemic rats with marked lymphocytosis (C1 to C5) had brighter gel bands intensity compared to the leukaemic rats with normal lymphocyte

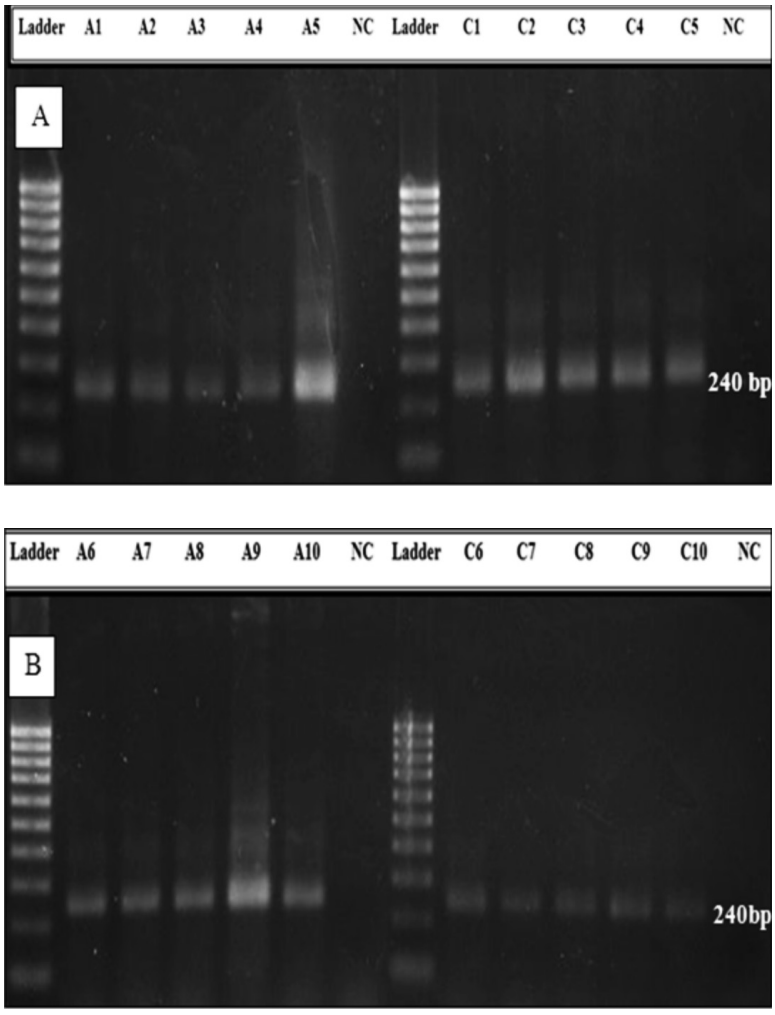

Fig. 2A and B: GAPDH gene amplification products (240 bp) of the control (A1, A2, A3, A4, A5, A6, A7, A8, A9, and A10) and leukaemic (C1, C2, C3, C4, C5, C6, C7, C8, C9, and C10) rats amplified using conventional RT-PCR. 100 bp DNA markers (Bioline, UK) are shown in lanes 1 and 8 for each picture. Negative controls (NC) show no amplification. Note that all RNAs extracted from the blood samples of the control and leukaemic rats had been successfully extracted

count (C6 to C10). This indicates that the expression of Bcl-2 gene was significantly higher in leukaemic rats with lymphocytosis. The results are consistent to the previous reports which demonstrated higher expression of Bcl-2 in patients diagnosed with high-grade breast cancer (Veronese *et al*., 1998), Burkitt lymphoma (Carbone *et al*., 2010) and advanced stage of colon cancer (Bousserouel *et al*., 2010).

In conclusion, Bcl-2 gene is highly upregulated in leukaemic rats with lymphocytosis, while EvaGreen qRT-PCR assay is highly sensitivity compared to conventional RT-PCR for the amplification of the Bcl-2 gene in leukaemic rats.

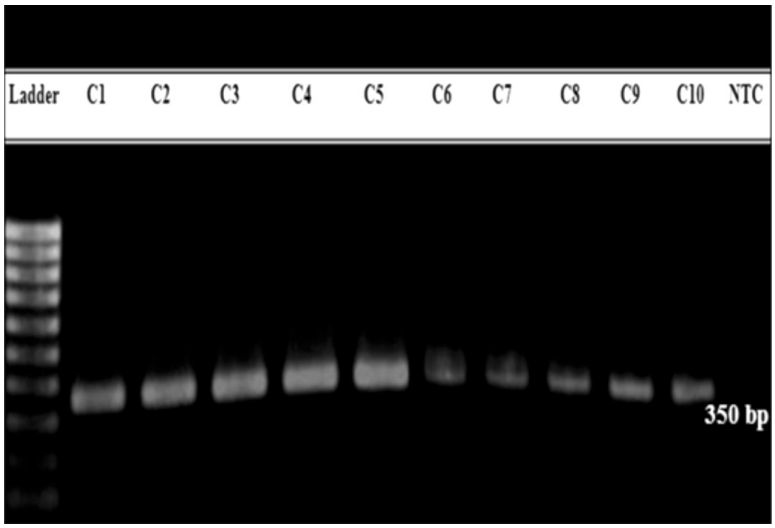

Fig. 3: Bcl-2 gene amplification products (350 bp) of the leukaemic rats amplified using EvaGreen qRT-PCR assay. 100 bp DNA marker (Bioline, UK) is shown in the first lane. Lanes C1 to C5 are the samples from rats with acute leukaemia, whereas lanes C6 to C10 are the samples from the rats with chronic leukaemia. The last lane is no template control (NTC). Note that brighter bands are observed in lanes C1 to C5 and faded bands are observed in lanes C6 to C10

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Expression of Circulating CD146 Associated with Endovascular Dysfunction in Adenine-induced Chronic Renal Damage in Rats Using an EvaGreen Real-time RT-PCR Assay

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ABSTRACT

The integrity of the endothelial layer of the blood vessel, intima, is critical since damage can lead to atherosclerosis. Assessment of its integrity is therefore very important although this can not be done through routine diagnostic analysis. An alternative non-invasive method should be established to assess its integrity. The objective of this study was to develop a quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) using an EvaGreen dye for the assessment of vascular endothelial damage-associated with chronic renal damage in rats via quantification of the expression level of CD146, which is one of the blood biomarkers of endovascular dysfunction and/or damage. Comparison of the sensitivity of the EvaGreen qRT-PCR and the conventional RT-PCR was also performed in this study. For this purpose, a total of 16 male Sprague Dawley rats were divided equally into two groups, namely the control and adenine. Results showed that rats fed with diet containing 0.75% adenine for six consecutive weeks developed chronic renal damage through elevations in the blood urea nitrogen (BUN) and blood creatinine (Cr) levels, increased urine protein to creatinine ratio (UPC) and decreased creatinine clearance (CrCl). The confirmatory diagnosis of chronic renal damage was made through gross and histopathological examinations of the kidneys. The results were consistent with lesions of chronic renal damage. The expression of circulating CD146 was mildly increased in rats with chronic renal damage analysed using the EvaGreen qRT-PCR or the conventional RT-PCR, suggesting a mild degree of endovascular damage. The intensity of the EvaGreen qRT-PCR products, however, was brighter than the conventional RT-PCR, indicating that the EvaGreen qRT-PCR is more sensitive compared to conventional RT-PCR, which is further recommended for analysis of CD146 expression in rats.

Keywords: Adenine, CD146, vascular dysfunction and damage, chronic renal damage, real-time RT-PCR, conventional RT-PCR

INTRODUCTION

The National Kidney Foundation Task Force on Cardiovascular Disease in Chronic Renal Disease reported patients with chronic kidney disease are at a higher risk of developing cardiovascular disease (CVD) (Levey *et al*., 1998). The evidence of the relationship between renal dysfunction and adverse cardiovascular (CV) events was first recognised in the dialysis

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patients who showed high incidence of CV death. Several studies have reported approximately 50% of individuals with end stage renal disease (ESRD) die from CVD-related events, such as heart attacks (Foley *et al*., 1998; Herzog *et al*., 1998; Tonelli *et al*., 2006). The development of CVD is suggested as the result of endothelial dysfunction and excessive oxidative stress. Endovascular dysfunction is an early feature of vascular disease in different diseases, such as chronic renal failure, diabetes, hypertension, hypercholesterolaemia, and coronary heart disease.

CD146, a membrane glycoprotein, is expressed in all human endothelium or vessel calibres. Expressed by the endothelial cells, it is one of the genes that is localised at the endothelial junction (Bardin *et al*., 1996; 2001). In blood, the vast majority of blood cells that express CD146 are lymphocytes (Duda *et al*., 2006). Hence, low level of the CD146 gene expression could be detected in healthy blood. Meanwhile, the increase in the number of circulating cells, including circulating endothelial cells (CECs) and lymphocytes expressing the membrane glycoprotein CD146, was observed in a wide variety of unhealthy conditions such as inflammatory, immune, infectious, neoplastic, and cardiovascular diseases which are likely to be evidence of profound vascular insult (Blann *et al*., 2005). Koc *et al*. (2005) reported that elevation of CEC numbers conveys a risk of future vascular events. Studies conducted by Bardin *et al*. (2003), Malyszko *et al*. (2004), Faure *et al*. (2006) and Sally *et al*. (2009) demonstrated that circulating CD146 and CD144 were significantly elevated in human patients with uraemic chronic renal failure. Those patients usually show various pathologies associated with endothelial dysfunction, such as acute coronary syndromes (Mallat *et al*., 2000), preeclampsia (Bretelle *et al*., 2003) or antiphospholipid syndrome (Dignat-George *et al*., 2004). The detection of circulating CD146 in those studies was performed via several means or methods such as ELISA, flow cytometry and real-time RT-PCR assays.

The objectives of this study were to develop a quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) using an EvaGreen dye for the assessment of vascular endothelial damage-induced by chronic renal damage in rats via quantification of the expression level of CD146, and to compare the sensitivity of the EvaGreen qRT-PCR and the conventional RT-PCR.

MATERIALS AND METHODS

Experimental Design

Both the control and Adenine groups equally comprised eight 6-week-old Sprague Dawley male rats. The rats in the control group were fed with normal rat pellets and water *ad libitum* throughout the 10-week experimental period. Meanwhile, the rats in the Adenine group were fed with ground rat pellets that were mixed with 0.75% Adenine (Sigma-Aldrich) and water *ad libitum* for 6 weeks (the treatment period), and this was continued with normal rat pellets and water *ad libitum* for 4 more weeks thereafter throughout the observation period. The rats were daily observed for clinical signs of renal failure.

Blood and Urine Analyses

Blood creatinine (Cr) and blood urea nitrogen (BUN) were analysed fortnightly during the treatment period. The blood samples were directly collected from the tail vein at weeks 0, 2, 4, 6, 8, and 10, and immediately analysed using an Automated Dry Chemistry Analyser (scil Reflovet® Plus, Roche Diagnostics) as per manufacturer's instruction. Both the blood and 12-hour urine samples were collected at the end of the experimental period. Urine volume was measured using a standard method. Serum Cr, urine Cr, and urine protein concentrations were analysed using an Automatic Biochemistry Analyser (Biorex, TRX7010, Manheim, Japan).

RNA Extraction from Fresh Whole Blood

The blood samples were taken from each rat via cardiac puncture at the end of the experiment.

RNA was extracted from the whole blood using a QIAamp RNA Blood Mini Kit from Qiagen (USA). The optical density (OD) of the extracted RNA was determined by measuring the absorbance at A260/A280 nm using a spectrophotometer (Eppendorf, German) and kept in -80°C until reverse transcription for cDNA synthesis.

Conventional RT-PCR Assay for Amplification of CD146 and GADPH Genes

The extracted RNA was converted into cDNA using a GeneAmp RNA PCR Core Kit (Applied Biosystems, USA), as per manufacturer's instruction. The cDNA products were stored at -20°C.

Master mix for the conventional PCR was prepared using sterile deionised water with the total reaction mixture of 20 uL containing 2 mM $MgCl_2$ solution, 1x PCR Buffer II, 200 uM for each dNTPs (Applied Biosystems, USA), 1.5 uM CD146/GAPDH forward and reverse primers (1st BASE, Malaysia), 0.05U AmpliTaq DNA polymerase (Applied Biosystem, USA), and 2 uL of sample containing 3 - 4 ng of cDNA. The amplification of the cDNA via conventional PCR was performed using a conventional PCR machine (Little Genius, BIOER). The amplification conditions were as follows: 1 cycle of 95°C for 2 minutes (predenaturation), followed by 30 cycles of 95°C for 30 seconds (denaturation), 51.2°C (for CD146) and 50.0°C (for GAPDH) for 30 seconds (annealing), 72°C for 30 seconds (extension), and 1 cycle at 72°C for 7 minutes for the final extension.

Meanwhile, the primers for the GADPH gene (designed using the Oligo Primer Analysis Software v.7) were: GAPDH forward: 5'-TGTGAACGGATTTGGCCGTA-3', and GAPDH reverse: 5'-CATTTGAT GTTAGCGGGATC-3'. The size of the amplification product is 240 bp (GenBank accession number: NM017008). All the CD146 amplification products were normalised with the GAPDH gene.

Real-time RT-PCR Assay for the Amplification of CD146 Gene

The same cDNA samples that were converted from the total RNA were used. Meanwhile, the master mix for the qPCR was prepared with the total reaction mixture of 20 uL containing 10 uL ImmoMix™ (Bioline, USA), 1 uL EvaGreen™ Dye (Biotium, USA), 1.5 um CD146 forward and reverse primers (1st BASE, Malaysia), as well 2uL of sample containing 3 - 4 ng of cDNA mixed with sterile deionised water in a single qPCR tube for each reaction. The amplification of CD146 gene was done using a real-time PCR machine (Biorad CFX96), with the following amplification conditions: 1 cycle of 94°C for 15 minutes (predenaturation), followed by 35 cycles of 94°C for 15 seconds (denaturation), 51.2°C for 30 seconds (annealing), 72°C for 45 seconds (extension).

The primers for CD146 gene used in this study were those previously described by Taira *et al*. (2004) and Hazilawati *et al*. (2010). The size of the PCR products is 100 bp.

Gel Electrophoresis

The PCR products were electrophoresed at 80V for 60 minutes in 1.5% agarose gel, stained with 0.5 ug/mL ethidium bromide solutions for 15-20 minutes, and visualised under UV transillumination (Alpha Imager, GEL DOC). The intensity of the bands of CD146 and GADPH in the control and Adenine groups was also compared.

Gross Lesion and Histopathological Examination

All the rats were sacrificed at the end of the 10-week experimental period. Both the left and right kidneys were collected and fixed in 10% formalin. The kidneys were processed with a standard protocol for Haematoxylin and Eosin (H&E) staining for histopathological examination.

Statistical Analysis

The results were analysed using SPSS version 17.0 for ANOVA and differences at $p<0.05$ were considered as significant.

RESULTS AND DISCUSSION

Blood Urea Nitrogen (BUN) and Blood Creatinine (Cr)

Figs. 1 and *2* show the blood urea nitrogen (BUN), and blood creatinine (Cr) concentrations of the control and Adenine groups throughout the 10-week experimental period. It was found that the baseline value for BUN and blood Cr concentrations of the control and Adenine groups were 9.33±0.57 mmoL/L and 7.97±0.63 mmoL/L, respectively, and 44.20±0.00 umoL/L for both groups. The rats in the Adenine group had gradual increase in the blood Cr and BUN concentrations throughout the treatment period. The highest values were observed at week 6; BUN was significantly increased to 38.05±7.11 mmoL/L and blood Cr was significantly increased to 201.30±43.85 umoL/L. Those parameters were decreased about half of the peak values during the observational period, although it was still significantly higher than the control group and/or normal ranges. A similar study conducted by Okada *et al*. (1999) showed Wistar rats were more susceptible to chronic renal failure induced by adenine. Blood Cr and BUN levels in their study were higher than those obtained in this study, whereby they reported 67% of the adenine-treated rats died of chronic renal failure during the study. In this study, the mortality rate was 0%.

Urine Protein to Creatinine Ratio (UPC) and Creatinine Clearance (CrCl)

Figs. 3 and *4* show UPC and CrCl of the control and adenine-treated rats. Even though BUN and blood Cr concentrations dropped during the observation period, albeit still significantly higher than the normal values, the UPC and CrCl results were consistent with the renal insufficiency. The UPC of the control rats was 3.38±1.61, while the UPC of the adenine-treated rats was 16.07±19.37, which was significantly five times higher than the control.

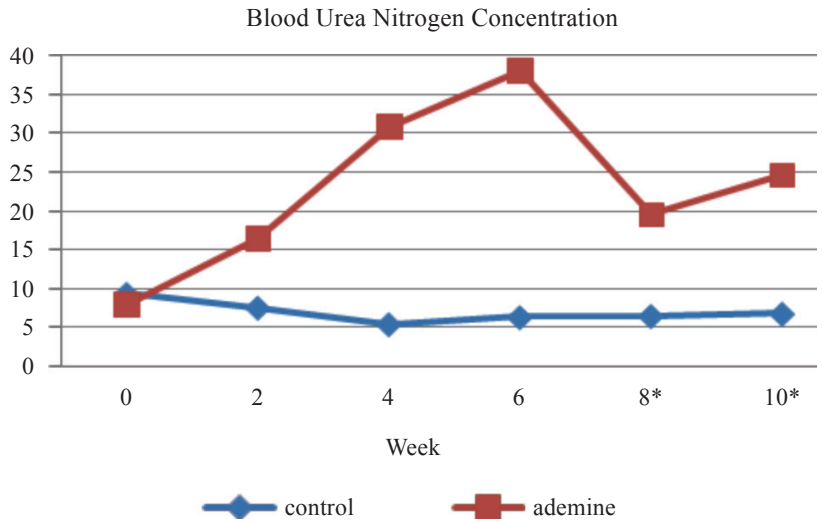

Fig. 1: The BUN concentrations of the Adenine group were gradually increased from week 0 to 6 (treatment period), and decreased at weeks 8 and 10 (observation period). The BUN results (weeks 2 to 10) for the rats in the Adenine group were significantly different at p<0.05 from those rats in the control group*

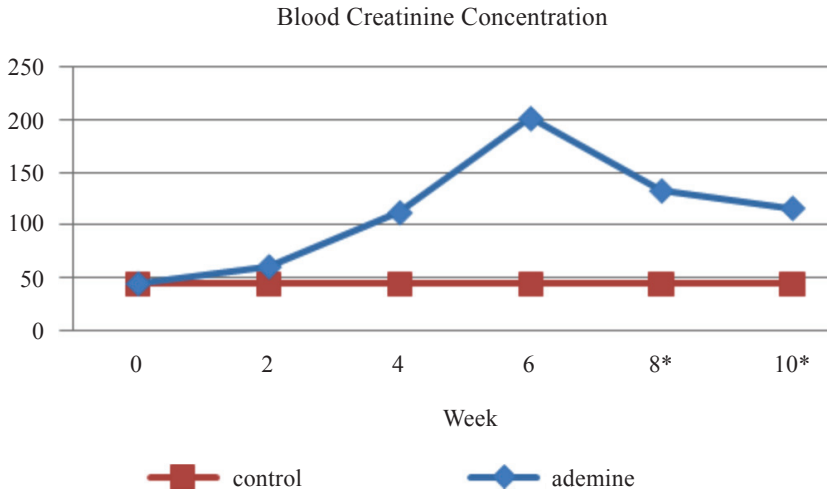

Fig. 2: The blood creatinine concentrations of rats in the Adenine group show a similar trend in their BUN concentrations. The results (weeks 4 to 10) were significantly different at $p<0.05$ from those rats in the control group

The CrCl for the control and adenine groups were significantly different, with 59.72±38.30 and 12.48±10.91 mL/hour, respectively. It was clearly showed that the rats in the Adenine group were unable to remove creatinine in the body as a result of the damage to the renal.

Detection of Circulating CD146 via EvaGreen qRT-PCR and Conventional RT-PCR

In this study, the transcription of CD146 in the blood was determined by both the qRT-PCR and conventional RT-PCR assays. Other than the comparison of the threshold cycle (Ct) from the amplification graph of the control and adenine groups, the intensity of CD146 bands from the

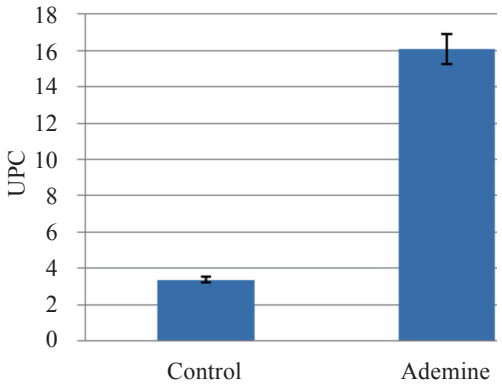

Fig. 3: The UPC of the rats in the Adenine group was significantly ($p<0.05$) increased five times higher than the control group

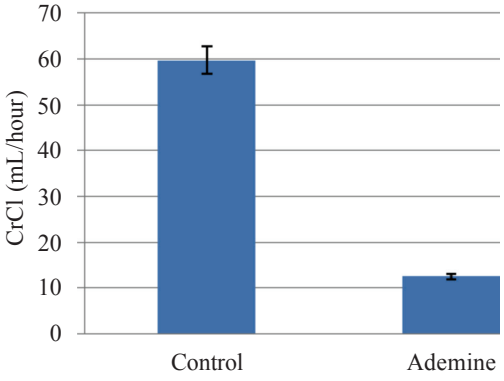

Fig. 4: The clearance of creatinine (CrCl) from the body through the urine of rats in the Adenine group was significantly ($p<0.05$) decreased compared to those rats in the control group. The results were comparable to the blood creatinine concentrations of the rats shown in Fig. 2

EvaGreen qRT-PCR and the conventional RT-PCR assays were also compared.

Figs. 5 and *6* show the amplification products of GAPDH and CD146 genes, respectively, from the control and adenine-treated rats amplified using the conventional RT-PCR. All the rats in both groups had expressed the GAPDH gene (*Fig. 5*). Meanwhile, the intensity of the bands was almost similar, indicating that the quality and quantity of the extracted RNAs were similar between the groups. The amplification of CD146 transcripts via the conventional method, however, was more difficult as both groups showed very faint bands, albeit the intensity of the bands was slightly brighter in the adenine group (*Fig. 6*).

Further investigation on the level of transcription of CD146 in the control and Adenine groups, via the EvaGreen qRT-PCR assay, revealed that the mean threshold cycle (Ct) of the adenine-treated rats was not significantly different from the control, 21.23 ± 0.38 (Adenine group) and 20.96 ± 0.79 (control group) (*Fig. 7*). The band intensity of the EvaGreen qRT-PCR products of the adenine-treated rats was slightly brighter and thicker than the control rats (*Fig. 8*), although the concentrations of the PCR products were almost comparable to the control (*Fig. 8*). This indicates that rats with chronic renal damage had a very mild increase in the expression of CD146 in the blood circulation, reflecting the dysfunction or damage of the endovascular wall. The findings of this study therefore suggested that the daily feeding of 0.75% adenine diet to rats for 6 weeks, followed by normal diet for 4 more weeks after that might have not induced significant damage to the endothelial layer of the blood vessels. Some previous studies reported that significant higher expression of CD146 has been observed in chronic renal failure patients analysed using ELISA (Baldin *et al*., 2003; Malyszko *et al*., 2004), flow cytometry (Faure *et al*., 2006) and also real-time RT-PCR assay (Sally *et al*., 2009).

A comparison of the intensity of bands between the conventional RT-PCR and the EvaGreen qRT-PCR assays clearly showed that the EvaGreen qRT-PCR assay is far more sensitive as compared to the conventional RT-PCR assay (*Figs. 6* and *8*). The results are in agreement with the findings reported by Di Trani *et al*. (2006) and Gurukumar *et al*. (2009) for the

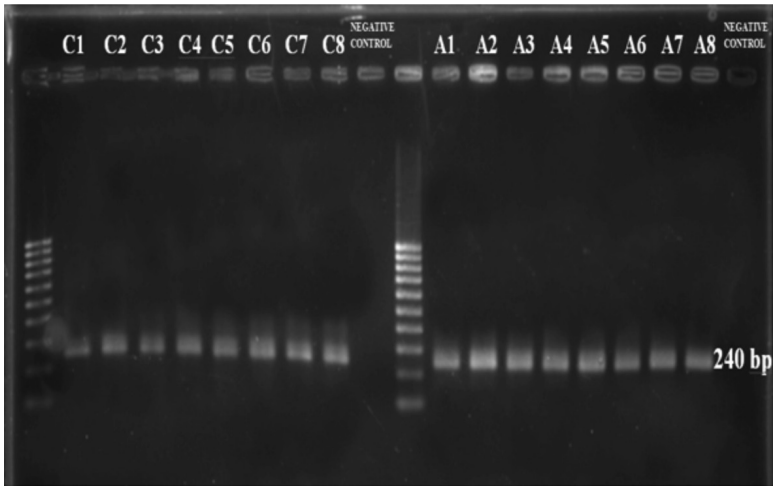

Fig. 5: Bands of the GADPH amplification products (240 bp) of the control (C1 to C8) and chronic renal damage rats (A1 to A8) amplified using the conventional RT-PCR assay. The amplications of the control rats are labelled as C1 to C8, whereas the amplications of the Adenine rats are labelled as A1 to A8. The DNA markers (Bioline, UK) are in lanes 1 and 11. Negative controls showed no amplification

detection of avian influenza and dengue viruses, respectively.

Gross and Histopathological Findings of the Kidneys

Fig. 9 shows gross and histopathological lesions of the kidneys of rats with chronic renal damages. All the rats that were treated with adenine had enlarged kidneys (*Fig. 9A[a]*) with generalised yellowish discolouration (stone like colour) of the renal parenchyma, which were consistent with severe renal damage. Microscopically, the kidneys had abnormal glomeruli (*Fig. 9B[c]*), crystal of adenine in the interstitial tissues (*Fig. 9B[d]*), infiltration of inflammatory cells in the interstitial tissues (*Fig. 9B[e]*), infiltration of inflammatory cells in the renal tubules (*Fig. 9C[f]*), necrotic cells (*Fig. 9D[g]*), tubular dilatation (*Fig. 9D[h]*), interstitial fibrosis (*Fig. 9E[i]*) and protein casts in the renal tubules (*Fig. 9F[j]*). The results were comparable to those of the previous reports (Yokozawa *et al*., 1986; Tamagaki *et al*., 2005).

Several studies, via ultrasound, vascular markers' measurements, vessel wall movement detector and occlusion plethysmography, have suggested that chronic renal failure is associated with impaired endothelial function (Guldener *et al*., 1998; Bolton *et al*., 2000). However, the precise mechanisms of chronic renal failure that induces endovascular dysfunction are still unclear. Several possibilities have been proposed, and these include oxidative stress-related accumulation of uraemic toxins and chronic inflammation (Stenvinkel *et al*., 1999). Other possible causes of endovascular dysfunction are accumulation of oxidised low-density lipoprotein (oxLDL) (Stenvinkel *et al*., 1999) and elevation of asymmetric dimethylarginine (ADMA), a competitive inhibitor of endothelial nitric oxide (NO) production (Zoccoli *et al*., 2001).

CONCLUSION

In conclusion, the study has demonstrated that circulating CD146 is mildly expressed in rats with chronic renal damage, and the EvaGreen qRT-PCR assay is more sensitive compared to conventional RT-PCR assay, and it is thus recommended for the analysis of CD146 expression in rats with endovascular dysfunction and/or damage.

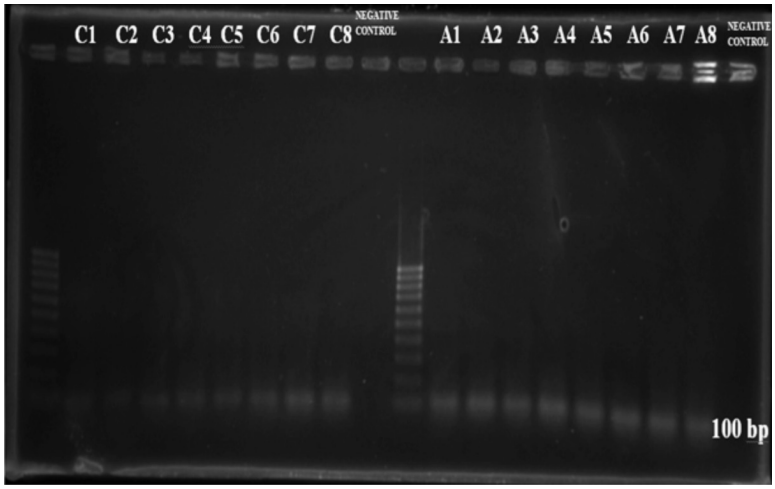

Fig. 6: Bands of CD146 amplification products (100 bp) of control (C1 to C8) and chronic renal damage rats (A1 to A8) amplified using the conventional RT-PCR assay. Amplicons of control rats are labeled as C1 to C8 and amplicons of Adenine rats are labeled as A1 to A8. DNA markers (Bioline, UK) are on lanes 1 and 11. Negative controls show no amplification

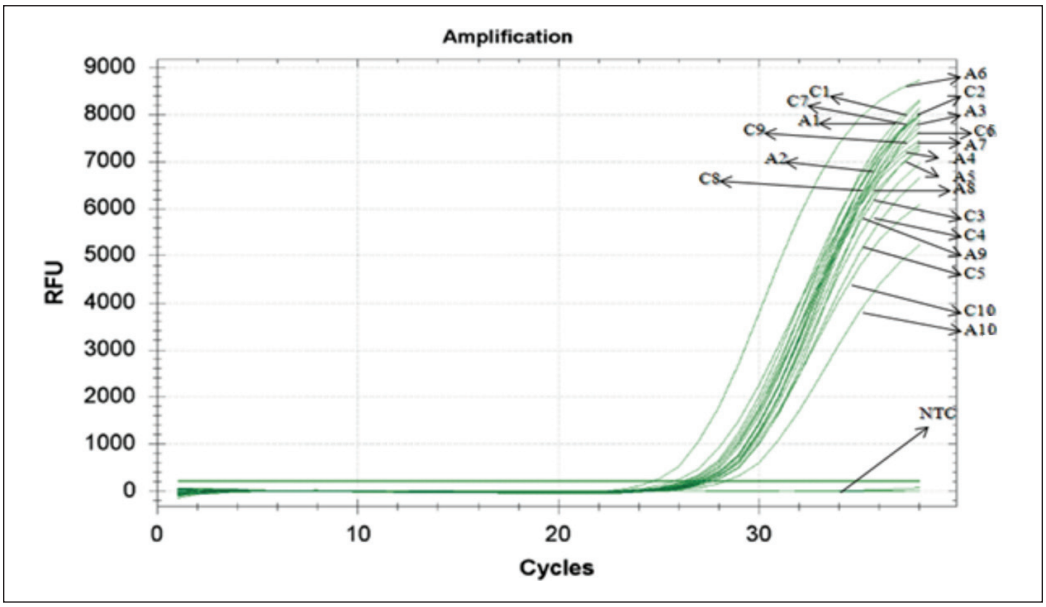

Fig. 7: Amplification graph of CD146 of the control (C1 to C8) and adenine-treated rats (A1 to A8). The melting curve peak for each sample clearly showed that all the amplified genes are products specific of CD146 with the melting temperature of 80.5°C±1.0°C

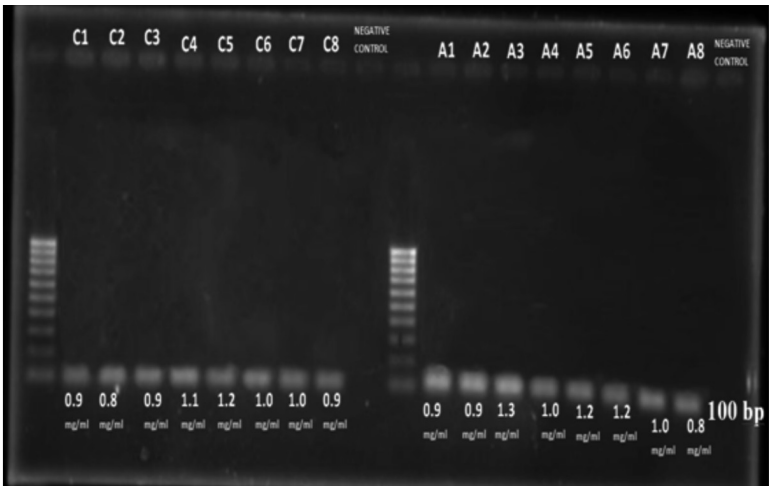

Fig. 8: Bands of CD146 amplification products (100 bp) amplified using the EvaGreen qRT-PCR assay. The amplifications of the control rats are labelled as C1to C8, whereas the amplications of the Adenine rats are labelled as A1 to A8. The DNA markers (Bioline, UK) are given in lanes 1 and 11. Negative controls show no amplification

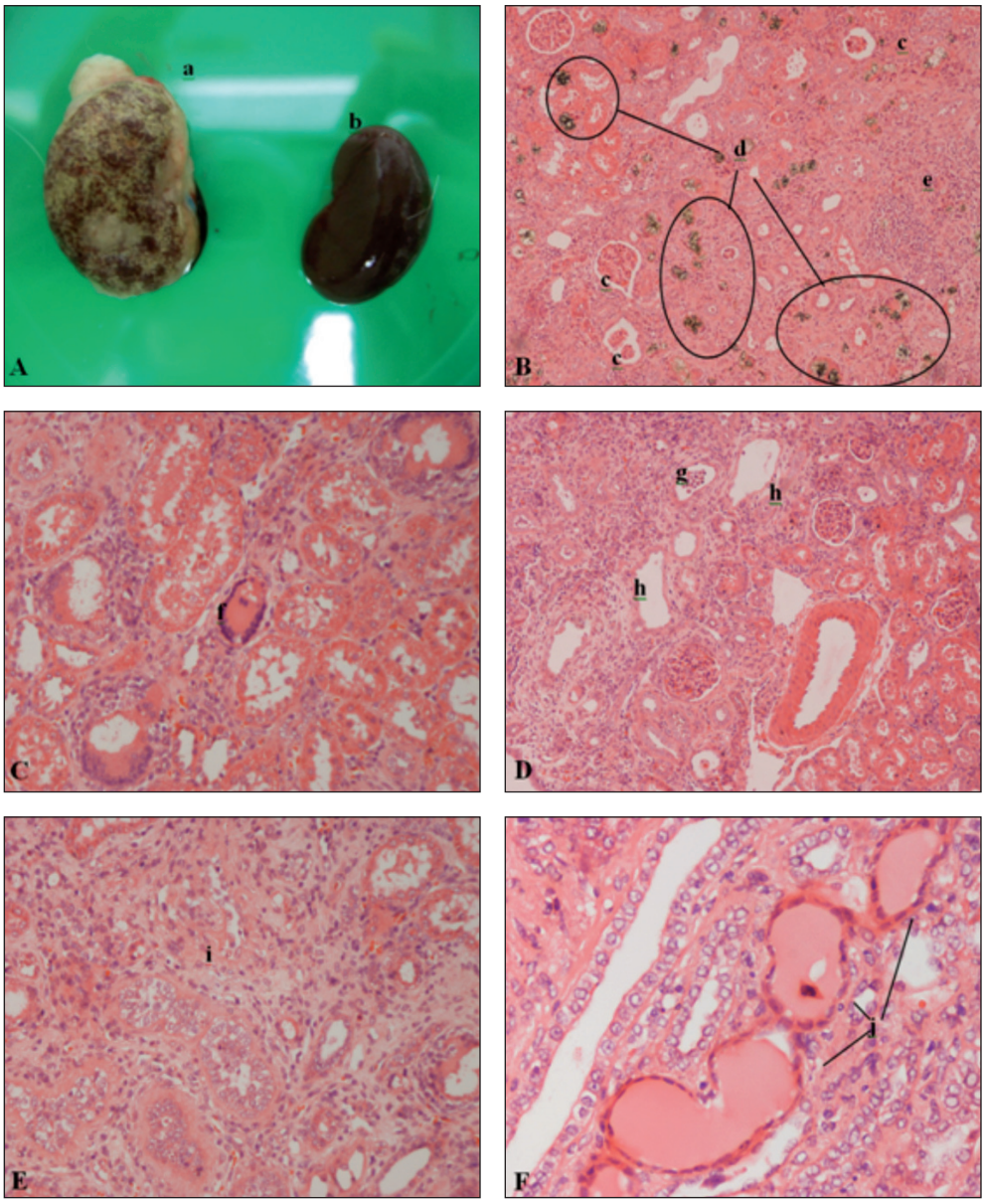

Fig. 9(A) a: Enlarged kidney with generalised yellowish discolouration, b: normal kidney; (B) c: abnormal glomeruli, d:adenine crystals, e: infiltration of inflammatory cells in the interstitial tissues; (C) f: infiltration of inflammatory cells in the renal tubule; (D) g:necrotic cells, h: tubular dilatation; (E) i: interstitial fibrosis; (F) j: protein casts

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